THE UNIVERSITY
of EDINBURGH

STUDIES TO INCREASE THE ACCURACY OF DIAGNOSIS AND PROGNOSTICATION FOR EQUINE GRASS SICKNESS

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Master of Science by Research

The University of Edinburgh

2016

The University of Edinburgh

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Degree Sought:	MSc by Research	*No. of words in the main text of Thesis:*	18,000
Title of Thesis:	STUDIES TO INCREASE THE ACCURACY OF DIAGNOSIS AND PROGNOSTICATION FOR EQUINE GRASS SICKNESS		

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Declaration

- This thesis contains original work and was composed entirely by the author.
- All the work described in the thesis and the included publication is the author's own, except for the following declarations:
  - The author performed histological analysis of all the rectal biopsy sections in the first part of the study (chapter 2), while Professor Bruce McGorum and BVM&S final year student Fionnuala Coyle analysed the ileal and cranial cervical ganglion sections, respectively.
  - Dr Ian Handel provided statistical analyses in the second part of the study (chapter 3). Specifically he constructed the percentage bodyweight changes for each time period, the receiver operating characteristic curves, the extrapolated data and the percentage survival prediction curves. The remaining statistical analyses were all performed by the author.
  - Bryony Waggett contributed to some preliminary data collection in the second part of the study (chapter 3) and the study described in appendix 1.
  - Professor Bruce McGorum and Dr Sandra Scholes captured histology photos
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  JAGO, R., HANDEL, I., HAHN, C., PIRIE, R., KEEN, J., WAGGETT, B. & MCGORUM, B. 2015. Bodyweight change aids prediction of survival in chronic equine grass sickness. *Equine Veterinary Journal,* 48**,** 792-797,
  is included in the appendices and incorporated into chapter 3. Permission was obtained from 'Equine Veterinary Journal' and a copyright agreement is included in the thesis. Contributions to this publication that were not the author's own work are summarised above.

Rachel Jago 5th December 2016

Abstract

Equine grass sickness (EGS) is a frequently fatal multi-system neurodegenerative disease, characterised by chromatolysis of the central, autonomic, enteric and somatic neurons of grazing equids.

An accurate, minimally invasive, ante-mortem, diagnostic test for EGS is currently lacking. While histological examination of haematoxylin and eosin stained rectal biopsies for chromatolytic neurons is insensitive as a diagnostic test for EGS, it was hypothesised that the diagnostic accuracy could be improved by immunolabelling for β-amyloid precursor protein (β-APP) which has increased expression in cranial cervical ganglia neuronal perikarya in EGS. The objective of the first part of the study was to develop a grading scheme for assessing the distribution and intensity of β-APP immunoreactivity within individual rectal submucosal neurons and subsequently to determine the diagnostic value of the distribution of different grades of neurons for EGS diagnosis. A retrospective case-control diagnostic accuracy study was performed. β-amyloid precursor protein immunoreactivity was increased in neuronal perikarya in biopsies of rectum from 21 EGS horses compared with those from 23 control horses. A weighted immunoreactivity grade exceeding 1.1 was 100% specific and sensitive for EGS, and the presence of at least one neuron with diffuse labelling of the entire cytoplasm (grade 3) was 95% sensitive and 100% specific for EGS. In conclusion, histological assessment of β-APP immunolabelled rectal biopsies is more accurate than conventional histological examination for EGS diagnosis. Further validation is required before this technique can be advocated for clinical decision making.

While acute and sub-acute EGS are invariably fatal, some chronic grass sickness (CGS) cases survive. Currently there are no objective criteria for predicting the outcome of CGS cases. The objective of the second part of the study was to determine whether the rate and/ or magnitude of bodyweight change of CGS cases during hospitalisation can provide an objective predictor of survival to discharge from hospital. A single centre retrospective observational study was performed by analysing the case records of all horses admitted for management of CGS to The Dick Vet Equine Hospital, University of Edinburgh between 1998 and 2013. The study sample comprised 213 horses, with 53.5% survivors (S) and 46.5% non-survivors (NS).

Compared with NS, S had significantly lower median maximum bodyweight loss as a percentage of first weight (S 5.9%, interquartile range 1.8-13.5; NS 12.7%, 6.4-17.3). Throughout all time periods analysed (0-7, 7-14, 14-21, 21-28, 0-14, 0-21 and 0-28 days from first weight recorded) S had significantly lower median bodyweight loss than NS, but no specific time period was more predictive of survival. Highest percentages of total bodyweight loss for individual horses were comparable for S (36%) and NS (37%). Survival prediction curves reporting percentage survival rates for all time periods analysed provided data to aid prediction of CGS survival. In conclusion NS had greater bodyweight loss than S. Rapidity and magnitude of bodyweight loss were equally predictive of outcome. Percentage survival prediction curves provide objective data to aid discussion of prognosis, but greater predictive specificity and sensitivity is required for clinical decision making in individual cases.

Lay summary

Equine grass sickness (EGS) is a disease of grazing horses which causes degeneration of nerves throughout the central and involuntary nervous systems, and particularly of the gastrointestinal tract. The majority of cases are fatal.

There is currently no accurate, minimally invasive, test to diagnose EGS in live animals. Standard microscopic examination of degenerating nerve cells in rectal biopsies is not a sensitive test for EGS diagnosis. It was hypothesised that the accuracy of using rectal biopsies in the diagnosis of EGS could be improved by labelling β-amyloid precursor protein (β-APP), a protein which accumulates within degenerating nerve cells. The aim of the first part of the study was to develop a grading scheme to assess the degree of β-APP labelling and subsequently determine if the distribution of different grades of labelling within nerve cells in rectal biopsies aids EGS diagnosis. β-APP labelling accumulated in nerve cells in rectal biopsies from 21 EGS horses compared with those from 23 control horses. An average grade of labelling allowed differentiation of all of the control and EGS horse samples. The presence of at least one nerve cell with generalised labelling allowed differentiation of 95% of the samples. In conclusion, labelling rectal biopsies with β-APP is more accurate than standard microscopic examination for diagnosing EGS. This technique needs to be tested in a greater number of cases before it can be used in real clinical cases.

A proportion of chronic grass sickness (CGS) cases, a milder form of the disease, can survive. However there are no objective criteria to help predict whether cases are likely to survive or require euthanasia on humane grounds. The aim of the second part of the study was to determine if the rate and magnitude of bodyweight change of CGS horses can predict which horses are likely to survive to discharge from the hospital. Horses admitted for management of CGS to The Dick Vet Equine Hospital, University of Edinburgh, between 1998 and 2013, were considered for inclusion in the study. Two hundred and thirteen horses were included in the study with 53.5% survivors (S) and 46.5% non-survivors (NS). On average NS lost a greater proportion of bodyweight than S, but this was not always the case for each individual horse. The highest percentage of bodyweight loss for individual horses were comparable for S (36%) and NS (37%), and consequently each case should be assessed on an individual case by

case basis. Curves predicting the likelihood of survival of CGS horses, based on the rate and magnitude of weight loss, were generated to provide data to help predict whether individual horses are likely to survive.

Dedication

For my Dad

Acknowledgements

I am enormously indebted to my principle supervisor, Professor Bruce McGorum, for being such an incredible supervisor, for all his endless inspiration, knowledge, help and never-ending support during these studies and throughout my senior clinical training scholarship.

I wish to express my sincere gratitude to Professor Scott Pirie for all his brilliant guidance, help and unerring support during these studies, and for providing samples and cases for both studies. Together with Dr John Keen, who also provided cases, I have the utmost admiration and respect for this amazing trio of medicine supervisors and have been very privileged to have been taught and guided by them these last four years.

I thank Dr Ian Handel for his endless help, patience and precise direction with the statistical analyses, and Dr Sandra Scholes for her invaluable knowledge, time and histopathological advice. I would also like to thank Dr Caroline Hahn for her contribution to study design for the second part of the study and Bryony Waggett for her contribution to data collection. Professor Geoffrey Pearson and Dr Tim Mair kindly provided samples for the first part of the study.

I thank Michael Algar and Joyce Wood, Animal and Plant Health Agency, for performing immunohistochemistry.

This work was generously part funded by the Equine Grass Sickness Fund. My senior clinical training scholarship and MSc fees were funded by the University of Edinburgh.

Thank you to all the clinicians and support staff at the Dick Vet Equine Hospital for their contribution to patient care and to the veterinary surgeons who referred the cases. I especially acknowledge the grooms and nurses who spent considerable time and effort nursing the patients.

Finally, I would like to thank my wonderful Mum who devoted her life to us as children and has supported me in every single step thereafter.

Abbreviations

AUC	area under the curve
β-APP	β-amyloid precursor protein
CCG	cranial cervical ganglia/ ganglion
CGS	chronic grass sickness
EGS	equine grass sickness
GIT	gastrointestinal tract
H&E	haematoxylin and eosin
ICC	interstitial cells of Cajal
IQR	interquartile range(s)
LMN	lower motor neuron(s)
ROC	receiver operating characteristic
NS	non-survivor(s)
S	survivor(s)

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1 Chapter One: Literature review

1.1 Introduction

Equine grass sickness (EGS), or equine dysautonomia, is a frequently fatal multi-system neurodegenerative disease, characterised by chromatolysis of the central, autonomic, enteric and somatic neurons of grazing equids. Disease severity is principally determined by the extent of neuronal degeneration in the submucosal and myenteric plexuses of the enteric nervous system, which, in the case of acute and subacute forms of the disease, is incompatible with life. Currently the only ante-mortem diagnostic test for EGS with high sensitivity and specificity is assessment of ileal histopathology, which requires invasive sampling; a less invasive method would clearly be desirable. Whilst a proportion of horses with chronic grass sickness (CGS) will survive, there are currently no objective criteria to predict survival of these cases. This literature review will discuss the scientific literature on EGS with an emphasis on current diagnostic techniques, histopathology and prognosis of the disease.

1.2 Clinical signs and disease sub-classification

Equine grass sickness presents as one of three clinical forms; acute, subacute and chronic. These categories are traditionally classified in the veterinary literature according to duration of survival; 1-2, 2-7 and >7 days, respectively (Hudson and Pirie, 2005, Lyle and Pirie, 2009, Pirie et al., 2014). However, this method of sub-classification, which relates exclusively to duration of survival, is somewhat arbitrary, with inevitable overlap between the three forms due to interventional factors (supportive care and elective euthanasia) which may affect duration of survival (Pirie et al., 2014). The extent of neuronal damage is of a continuum scale and the sub-classifications should also be thought of as such, with some overlap observed (Pirie, 2002). The clinical sub-classifications should be based on the nature and progression of clinical signs (Pirie et al., 2014), both of which correlate with the extent of neuronal damage (Pogson et al., 1992, Doxey et al., 1992, Scholes et al., 1993b, Doxey et al., 1995b).

Correctly sub-classifying EGS is important with regard to prognosis. Acute and subacute forms of the disease are considered to be invariably fatal and treatment is not recommended (Pirie et al., 2014). Some cases of CGS survive with appropriate nursing care (Milne et al., 1994, Doxey et al., 1995a, Doxey et al., 1998, Doxey et al., 1999).

From a clinical perspective, the EGS disease phenotype principally reflects dysfunction of the parasympathetic neurons in the enteric nervous system, with the remainder of clinical signs attributable to the dysfunction of remaining autonomic and somatic neurons (Scholes et al., 1993b, Hahn, 2000, Hahn et al., 2001). All sub-classifications of the disease may demonstrate signs of dullness, anorexia, oral, pharyngeal and oesophageal dysphagia, salivation, colic, reduced gastrointestinal motility, tachycardia, bilateral ptosis, patchy sweating and muscle fasciculations, predominantly of the triceps and quadriceps (Hudson and Pirie, 2005, Wylie and Proudman, 2009, Lyle and Pirie, 2009). The severity of these signs often correlates with the sub-classification of disease (Pirie et al., 2014), e.g. the magnitude of tachycardia and intestinal dysmotility is greater in acute than subacute and chronic forms of the disease.

In addition to the above clinical signs, chronic cases are usually characterised by profound weight loss (McGorum and Kirk, 2001), with a characteristic 'tucked up' abdominal silhouette. The magnitude of weight loss appears greater than that which can solely be attributed to a lack of food intake and cachexia is suspected (McGorum and Kirk, 2001). Progressive generalised myasthenia occurs, as demonstrated by a base-narrow 'elephant on a tub' stance, leaning against walls, weight shifting between limbs and increased periods of recumbency (Lyle and Pirie, 2009, Pirie et al., 2014). In contrast to cases of neuroparalytic botulism, the muscle fasciculations observed whilst standing often persist during periods of recumbency and it is speculated that other factors, in addition to weakness, may contribute to the fasciculations (Pirie et al., 2014). Rhinitis sicca is considered to be pathognomonic for the disease, and may cause nasal obstruction and respiratory noise in the chronic form of the disease (Prince et al., 2003). Examination *per rectum* often reveals dry faecal pellets coated with inspissated mucus in all forms of the disease (Pirie et al., 2014). The main differential diagnoses for CGS include botulism, equine motor neuron disease, hypocalcaemia and other causes of dysphagia and weight loss (Lyle and Pirie, 2009).

The firm and corrugated secondary large colon and caecal impactions present in subacute and some acute cases differentiate this category from the chronic form and reflect the moderate ileus present (Lyle and Pirie, 2009). The predominant differential diagnosis for subacute EGS is a primary large colon impaction, an unusual finding in a grass kept horse, or impactions that occur as a secondary consequence of other primary conditions, such as large colon displacement.

Acute EGS cases have marked acute gastrointestinal ileus with gastric and generalised small intestinal distension and subsequent high volume malodorous gastric reflux, which may spontaneously reflux via the nares (Pirie et al., 2014). The primary differential diagnosis for acute cases is a strangulating or non-strangulating small intestinal obstruction (Lyle and Pirie, 2009). Apart from a degree of haemoconcentration, cases of EGS have cardiovascular stability, despite being markedly tachycardic (typically 70-120 beats/ min). The relative absence of abdominal pain in acute EGS may aid differentiation from surgical colic cases.

1.3 Epidemiology

The first case of EGS was reported in Angus, Scotland, in 1909 (Greig, 1942). Equine grass sickness has since been reported throughout the UK and northern Europe, including France (Lhomme et al., 1996), Belgium (Christmann et al., 1999), the Netherlands (Leendertse, 1993), Germany, Switzerland (Eser et al., 2000), Denmark (Bendixen, 1946), Hungary (Schwarz et al., 2012), Austria (Wlaschitz and Url, 2004), Sweden (Obel, 1955) and the Czech Republic (Melkova et al., 2014). The first histopathologically confirmed case of EGS in southern Europe occurred in Cyprus (Protopapas et al., 2012). Confirmed cases have also been reported in the Falklands (Woods and Gilmour, 1991), Australia (Stewart, 1977), and a single case in a mule in the United States (Wright et al., 2010). A condition, clinically and pathologically, indistinguishable from EGS called ‘mal seco’ (dry sickness) is recognised in Argentina, the Falklands and Chile (Uzal et al., 1994, Uzal and Robles, 1993, Uzal et al., 1992, Araya et al., 2002). Further histopathological investigations are necessary to determine whether the disease called ‘tambora’ occurring in Columbia is the same disease (Oliver-Espinosa, 2008).

Equine grass sickness affects all equidae including donkeys (Wylie et al., 2011, Mellor et al., 2013), mules (Wright et al., 2010), zebras (Ashton et al., 1977) and Przewalski's horses (Ashton et al., 1977, Girling et al., 2015). The most recent overall median incidence rates for EGS, on premises having at least one previous incident of EGS, is reported as 2% annually (Newton et al., 2004, Ireland et al., 2011). The incidence is highest in East Scotland (Wood et al., 1998, McCarthy et al., 2001, Wylie et al., 2011).

Whilst the aetiological agent of EGS remains unknown, recent epidemiological investigations have identified multiple risk factors, suggesting a multifactorial aetiology is most likely.

1.3.1 Horse related risk factors

1.3.1.1 Age

Whilst the disease has been diagnosed in horses from 12 days (McGorum et al., 2003) to 47 years of age (Wylie et al., 2011), younger horses have consistently been reported as being at greater risk of EGS. The largest case-control study examining signalment reported the highest incidence of EGS in two year olds (Wylie et al., 2014) whilst other studies consistently found younger mature horses (3 to 6 years) to be at greater risk (Doxey et al., 1991a, Wood et al., 1998, McCarthy et al., 2004, Wylie et al., 2011). It is speculated that the association of EGS with particular age categories may reflect different levels of protective passive and acquired immunity and/ or tolerance to the causal agent (Wood et al., 1998, McCarthy et al., 2004, Wylie et al., 2014).

1.3.1.2 Breed

Two studies found certain breeds to have a greater association with the disease following univariable analyses (Wood et al., 1998, McCarthy et al., 2004), however these associations were not significant after multivariable analyses. Native Scottish breeds compared to crossbreds were reported in a single study to be at increased risk in both uni- and multivariable analyses (Wylie et al., 2014). Other studies have reported breeds with an increased/ decreased chance of survival (Milne et al., 1994, Doxey et al., 1998), however these findings are inconsistent, with other studies failing to identify any breed-associated differences in survival (Doxey et al., 1991a).

1.3.1.3 Gender

Wylie et al. (2014) reported stallions to be associated with lower odds of developing EGS compared with mares, which contrasts with an earlier study (Wood et al., 1998) that reported females less likely to be affected. The majority of studies have found no gender predispositions to EGS (Doxey et al., 1991a, Wylie et al., 2011).

1.3.1.4 Body condition

Doxey et al. (1991a) found 95% of EGS cases to be in good/ fat bodily condition compared to 52% of the control population, however, the control data were collected at a different time of the year. More recently, Wood et al. (1998) reported that horses in good condition were not at any greater risk.

1.3.1.5 Immunological status

During investigations to determine if non-fatal exposure to the causative agent of EGS induces protection, Wood et al. (1998) reported that horses with a history of prior contact with an EGS case were associated with an approximately 10-fold reduced likelihood of disease. Furthermore, McCarthy et al. (2004) found that increasing serum antibody titres to *Clostridium botulinum* type C, C. *botulinum* C1 neurotoxin and C. *novyi* type A surface antigens were significantly associated with a decreased risk of EGS.

1.3.2 Climate related risk factors

1.3.2.1 Seasonal incidence

Although EGS cases have been reported as occurring in every month of the year (Wylie et al., 2011), the disease has a clear seasonal occurrence. Consistent with the Veterinary Clinical Observation Unit (1970) grass sickness survey report for Scotland in which 40% of cases occurred in May, Doxey et al. (1991a) reported higher incidences between April and July, with a principal peak in May. A study over 10 years (Wylie et al., 2011) reported 60.6% of cases occurred during April, May and June, with the fewest cases in January. Early studies have reported a second peak of EGS cases in autumn in Scotland (Doxey et al., 1991a), but this has not been

subsequently confirmed in nationwide surveillance reports (Wylie et al., 2011). It is likely that geographically localised trends in occurrence have been lost in the nationwide averages reported by Wylie et al. (2011).

1.3.2.2 Weather patterns

It has been reported that the majority of outbreaks occur during cooler, drier weather associated with irregular ground frosts (Doxey et al., 1991b). Wood et al. (1998) reported that 66% of cases were preceded by 2 weeks of predominantly dry weather.

The largest case-control study of EGS to date confirmed a number of meteorological related risk factors associated with increased risk of EGS; lower average temperature (average difference 0.08-0.13ºC), greater sunshine hours (average difference 0.01-0.05 hours) and frost days (average difference 0.05-0.2 days) (Wylie et al., 2014). Whilst the magnitudes of the average differences were not large, some associations were consistent with previous reports (Doxey et al., 1991b, Wood et al., 1998). However, unlike the earlier reports that identified a short-term temporal association, Wylie et al. (2014) only identified statistically significant differences for data over longer time periods (e.g. average frost days was only statistically significant if 6 or more months were averaged).

1.3.3 Premises related risk factors

1.3.3.1 Type of establishment

Whilst specifically analysing the type of equestrian establishment by the type of work undertaken, Doxey et al. (1991a) failed to identify a correlation between the type of work and incidence of EGS. In contrast, Newton et al. (2004) found an increased rate of EGS recurrence in stud farms and riding establishments in univariable but not multivariable analyses.

1.3.3.2 Soil type

Loam and sandy soils were associated with an increased risk of recurrence of EGS and chalk soils with decreased recurrence (Newton et al., 2004). It was speculated that as sand and loam soils may be more easily disturbed and turned over, soil inhabitants, such as earthworms and moles, are able to burrow freely, causing disruption and

increasing the rate of contamination of grass with soil borne microorganisms (Newton et al., 2004).

1.3.3.3 Previous occurrence of equine grass sickness

Animals on premises where EGS had previously occurred within the last two years were at increased risk of EGS (Wood et al., 1998). Wylie et al. (2011) found most cases reported from England and Wales occurred on premises that had not previously had a case of EGS, while in Scotland most cases were reported from premises that had a case of EGS previously.

1.3.4 Management related risk factors

1.3.4.1 Grazing

As the name suggests, there is a strong association between the development of EGS and grazing (Wood et al., 1998). Both Doxey et al. (1991a) and Wood et al. (1998) identified a significantly higher incidence of EGS in horses grazing full time compared to part time. Whilst rare cases have been reported in horses entirely stable kept these were either not histologically confirmed (Forsyth, 1941, Wood et al., 1998) or had only been stabled for 1 week (Wood et al., 1998).

1.3.4.2 Dietary

Earlier studies failed to identify a relationship between supplementary feeding or stage of pasture growth and EGS (Doxey et al., 1991a, Wood et al., 1998), but more recently, the feeding of hay or haylage has been associated with a decreased risk of disease (McCarthy et al., 2004). McCarthy et al. (2004) also reported an increased risk with changing of feed type or quantity during the 14 days prior to disease onset.

1.3.4.3 Movement/ stress

Animals that had recently moved fields, especially within the preceding 2 weeks, had the largest effect in a multivariate model (odds ratio 29.7) (Wood et al., 1998). Of 218 EGS cases identified in a retrospective survey, 45% had moved from another field or premise in the two weeks prior to illness with 12% incurring a stressful event such as

castration, foaling and breaking in the two weeks preceding onset of illness (Doxey et al., 1991a).

1.3.4.4 Anthelmintic use

In comparison to horses receiving anthelmintics ≥ 6 monthly, horses receiving an anthelmintic at any greater frequency had an increased risk of recurrence of EGS (Newton et al., 2004). The use of ivermectin at both the ultimate and penultimate treatments has been associated with an increased risk of EGS (McCarthy et al., 2004).

1.3.4.5 Removal of faeces

In one study, the manual removal of horse faeces from pastures significantly reduced the risk of recurrence while mechanical removal increased the risk of recurrence of EGS (Newton et al., 2004). Again, it was speculated that mechanical removal of faeces (e.g. sweeping the paddocks) may disturb the soil surface and possibly disseminate the causal agent.

Whilst multiple risk factors have been identified in both univariable and multivariable analyses, only a few risk factors have been consistently reported in the literature (grazing, age, time of year and recent movement to new pasture). These epidemiological findings give insight into the aetiology of the disease and possible protective control measures.

1.4 Aetiology

As the name suggests, the disease almost exclusively affects grazing equids and epidemiological studies support the role of an ingested soil-borne agent, which in certain circumstances, produces a putative neurotoxin (Pirie et al., 2014). Whilst the aetiopathogenesis is yet to be elucidated, likely causes include toxico-infection with *Clostridium botulinum* types C or D (Tocher et al., 1923, Hunter et al., 1999, McCarthy et al., 2004, Ireland, 2014) and pasture derived mycotoxins (Doxey et al., 1991b, Robb et al., 1997).

Botulism was first proposed as the underlying cause of EGS as early as 1918 when post-mortem studies isolated bacteria with morphological and toxicity characteristics similar to *C. botulinum* (Tocher et al., 1923). A subsequent vaccination field trial using antitoxin-neutralised *C. botulinum* successfully reduced the incidence of EGS in vaccinated horses compared with those receiving placebo (Tocher et al., 1923). However, the validity of the study was questioned, and funding was directed elsewhere (Newton et al., 2010), until recently when the botulinum theory has been re-investigated. Whilst an association between *C. botulinum* type C within the intestinal tract and EGS has been confirmed (Hunter et al., 1999), a causal relationship has not been proven. Furthermore, a similar difference in detection frequency of C. *perfringens* between EGS and controls has been reported, potentially reflecting a generalised clostridial overgrowth in the small intestine of EGS horses secondary to pre-existing gastrointestinal dysmotility (Waggett et al., 2010b). McCarthy et al. (2004) found that increasing serum antibody titres to C. *botulinum* type C, C. *botulinum* C1 neurotoxin and C. *novyi* type A surface antigens were significantly associated with a decreased risk of EGS, further evidence of an association between *C. botulinum* and EGS. The outcome of the ongoing nationwide triple blinded randomised placebo controlled trial of *C. botulinum* type C toxoid vaccination will support or refute the theory that *C. botulinum* has an obligatory role in the aetiology of EGS (Ireland, 2014, Ireland et al., 2016).

Whilst the particular climatic conditions which often precede occurrences of EGS have been considered to be consistent with the involvement of a pasture derived mycotoxin (Doxey et al., 1991b), all studies to date have failed to associate any particular fungal species with EGS (Doxey et al., 1990). *Fusarium* spp. which have neurotoxic properties *in vitro*, have been isolated from EGS pastures (Robb et al., 1997). Further investigations into the potential role of pasture mycotoxigenic fungi, utilising modern molecular techniques, are currently ongoing (B. C. McGorum, personal communication).

Other possible aetiologies of EGS that have been discounted include cyanogenic wild white clover (*Trifolium repens*) (Tocher, 1924, McGorum and Anderson, 2002,

McGorum et al., 2012), alsike clover (*Trifolium hybridum*) (Tocher et al., 1923), insect vectors (Lloyd, 1934) and niacin deficiency (McGorum et al., 2016a).

1.5 Pathology

Equine grass sickness is a polyneuropathy affecting the central and autonomic nervous systems, with pathological lesions most evident in the paravertebral, prevertebral and enteric (submucosal and myenteric) ganglia (Cottrell et al., 1999). The histological feature of EGS, first described in 1955, is neuronal degeneration with extensive chromatolysis (Obel, 1955), with loss of Nissl substance, cytoplasmic vacuolation, eccentric nuclei, nuclear pyknosis, nuclear karyorrhexis, eosinophilic spheroids and axonal dystrophy (Obel, 1955, Gilmour, 1975, Scholes et al., 1993b, Whitwell, 1997). Neuronal cell death ensues, followed by neuronophagia with a subsequent reduction in the number of neurons (Obel, 1955, Brownlee, 1965, Scholes et al., 1993b). Both cytoplasmic vacuolation and eccentric nuclei can occur as a normal variation, with the former also occurring as a processing artefact (Scholes et al., 1993b).

1.5.1 Enteric nervous system

The enteric nervous system comprises the submucosal and myenteric plexuses. The submucosal plexus is located in the submucosa between the muscularis mucosae and the muscularis externa, giving rise to postganglionic neurons innervating the secretory epithelium, blood vessels and smooth muscle of the muscularis mucosae (Furness and Costa, 2006). Two separate submucosal plexuses have been identified with an internal (Meissner's) plexus, consisting of smaller ganglia (up to four neurons) adjacent to the muscularis mucosae and an external (Henle's) plexus, consisting of larger (up to 20 neurons) ganglia, adjacent to the circular muscularis (Scholes et al., 1993b, Pearson, 1994). This is consistent throughout the small and large intestine, but in the rectum, most submucosal ganglia are situated within the inner plexus with adipose tissue occupying the outer submucosa (Scholes et al., 1993b). The myenteric (Auerbach) plexus is located between the inner circular and outer longitudinal muscularis layers and primarily controls gastrointestinal motility (Furness and Costa, 2006).

Several studies have reported degeneration and depletion of enteric neurons in cases of EGS (Obel, 1955, Barlow, 1969, Pogson et al., 1992, Doxey et al., 1992, Scholes et al., 1993b, Doxey et al., 1995b). Neuronal pathology is not uniform throughout the gastrointestinal tract (GIT) and Scholes et al. (1993b) were the first to compare severity of pathology at different sites. The distribution of neuronal degeneration and neuronal depletion was widespread throughout the duodenum, jejunum, caecum, large and small colon in acute cases, but only the ileum had consistently severe pathology in CGS (Scholes et al., 1993b). With more objective data, Doxey et al. (1995b) confirmed this finding, reporting that overall the ileum had significantly greater neuronal depletion and proportion of chromatolytic neurons in all three forms of the disease, compared to the jejunum and small colon. However, in a minority of acute and subacute cases, the most severely affected region was the jejunum (Doxey et al., 1995b). More recently, Milne et al. (2010) reported the ileum to consistently be more severely affected than the jejunum in 23 cases of EGS.

Histological examination of the jejunum, however, may facilitate more accurate differentiation of the sub-classifications of EGS, since the proportion of chromatolytic neurons and the depletion of neurons is greater in the jejunum of acute and subacute EGS than in CGS (Pogson et al., 1992, Doxey et al., 1992, Doxey et al., 1995b). Although the same pattern is evident in the small colon, it is consistently less obvious at this site, compared with the jejunum (Doxey et al., 1995b). This contradicts earlier findings by Barlow et al. (Barlow, 1969) who reported a greater degree of neuronal depletion in chronic cases, however only 3 chronic cases were compared, and the number of neurons was only commented upon subjectively.

Varying degrees of neuronal pathology are described in the stomach and rectum of acute cases, while gastric and rectal neurons of chronic cases have a similar appearance to controls (Scholes et al., 1993b). Scholes considered that full thickness rectal biopsies may only give a positive diagnosis in a proportion of acute cases (Scholes et al., 1993b).

The numbers of ganglia and total neurons were greater in the submucosal plexus compared to the myenteric plexus (Doxey et al., 1992, Doxey et al., 1995b) having 2-3 times more neurons per section (Doxey et al., 1995b). This could have potential

practical advantages when assessing more superficial mucosal/ submucosal biopsies. Whilst the submucosal plexus was more severely affected in the ileum (Doxey et al., 1995b, Milne et al., 2005), the myenteric plexus was more severely affected in the jejunum (Pogson et al., 1992, Doxey et al., 1992, Doxey et al., 1995b). The distribution of neuronal damage is uniform within each region of the intestine, throughout the jejunum, ileum and small colon (Doxey et al., 1995b).

The interstitial cells of Cajal (ICC), the intrinsic pacemakers of the smooth muscle of the intestinal tract (Sanders et al., 2012), are present in reduced numbers in the ileum and pelvic flexure of horses with EGS (Hudson et al., 2001).

1.5.2 Autonomic ganglia

In addition to the enteric nervous system, neuronal degeneration and depletion is evident elsewhere throughout the autonomic nervous system, including the following paravertebral ganglia: cranial cervical, caudal cervical and stellate ganglia, and the thoracic and abdominal sympathetic trunk. Prevertebral ganglia reported to be affected include the cranial (coeliac) mesenteric, caudal mesenteric and the parasympathetic terminal cardiac ganglia (Obel, 1955, Brownlee, 1959, Barlow, 1969, Gilmour, 1975, Pogson et al., 1992, Doxey et al., 1992, Griffiths et al., 1993, Perkins et al., 2000, John et al., 2001, Shotton et al., 2011).

For all sub-classifications of the disease, the proportion of chromatolytic neurons in the paravertebral ganglia is significantly increased compared with control horses, and the percentage of chromatolytic neurons increases with the severity of disease (Pogson et al., 1992, Doxey et al., 1992). Whilst Pogson et al. (1992) reported that the cranial mesenteric ganglia were not as severely affected as cranial cervical ganglia (CCG) in acute and subacute EGS, Shotton et al. (2011), utilising immunofluorescence labelling techniques, found evidence of greater neurodegeneration in the cranial mesenteric ganglia than the CCG, perhaps indicating a greater vulnerability of the prevertebral neurons to the putative neurotoxin.

In contrast to enteric ganglia, multiple studies have demonstrated that the total number of neurons is significantly reduced in the autonomic ganglia of CGS, compared to acute and subacute EGS (Pogson et al., 1992, Doxey et al., 1992). It was speculated

that neuronal depletion occurs in the jejunum of acute cases before neuronal loss has occurred in the peripheral ganglia (Pogson et al., 1992).

No significant differences were demonstrated in the proportion of chromatolytic neurons throughout different sections of individual paravertebral ganglia, nor between the left and right CCG, while the cranial mesenteric ganglia had a patchy distribution of chromatolytic neurons (Pogson et al., 1992). This is relevant when collecting samples for histopathological assessment.

1.5.3 Central nervous system

Whilst often termed 'equine dysautonomia', the evidence for involvement of somatic lower motor neurons (LMN) indicates that EGS is more accurately termed as a multisystem disease (Barlow, 1969, Gilmour, 1973, Hahn, 2000). Neuronal chromatolysis was most consistently noted in the LMN of general visceral efferent nuclei of cranial nerves III and X, and the general somatic efferent nuclei of cranial nerves III, V, VII and XII (Hahn et al., 2001). Chromatolysis was also reported in 83% of the intermediolateral horn LMN and 35% of ventral horn LMN in the spinal cord of EGS cases (Hahn et al., 2001).

Despite the severe chromatolysis of ventral horn LMN, there was no evidence of neuronal cell death, axonal pathology or muscle denervation or re-innervation, such as muscle fibre type grouping, in somatic muscles innervated by chromatolytic neurons (Hahn, 2000). Therefore, in contrast to autonomic ganglion neurons and enteric neurons, it appears that chromatolytic central neurons may not progress to cell death in EGS and thereby could potentially recover with time. Consistent with this possibility, histological assessment of an animal recovered from EGS identified no pathological changes in the central nervous system or striated muscle (Milne et al., 2005).

1.5.4 Gross post mortem findings

Gross post mortem findings are largely reflective of the effects of dysautonomia on the GIT (Pirie et al., 2014). Acute EGS cases generally have gastric and small intestinal fluid distension (Lyle and Pirie, 2009), and possibly gastric rupture and associated peritonitis. Firm and corrugated large colon and caecal impactions are present in

subacute and some acute cases, with a black coating adhered to the colonic mucosa (Pirie, 2006). Chronic EGS cases have a relatively empty GIT, evidence of cachexia, rhinitis sicca (Lyle and Pirie, 2009) and possibly aspiration pneumonia. All cases can have evidence of reflux oesophagitis, with linear erosions of the distal oesophagus (Pirie, 2006). Dry, firm mucus coated faecal balls are also found in the small colon and rectum of the majority of subacute and chronic cases (Pirie et al., 2014).

1.6 Ante-mortem diagnosis of equine grass sickness

Reaching a definitive ante-mortem diagnosis of EGS together with appropriate disease sub-classification is not only important for the individual's prognostication and for consideration of treatment options, but also for provision of appropriate advice to allow early implementation of management changes for other at-risk co-grazing horses.

An ante-mortem diagnosis of EGS can be made presumptively, after considering the nature and progression of clinical signs, subjective ancillary tests, history and epidemiological factors, together with the exclusion of differential diagnoses that may mimic EGS clinically (Pirie et al., 2014). Milne et al. (1994) and Doxey et al. (1995a) found the accuracy of diagnosis of CGS to be 100% on the basis of clinical signs alone. However, these clinical evaluations were performed by veterinary surgeons at the University of Edinburgh, who have considerable experience in the diagnosis of EGS. Whilst the exposure to clinical cases is much higher for veterinary surgeons in eastern Scotland (Wood et al., 1998, McCarthy et al., 2001, Wylie et al., 2011), some cases referred to the author with a presumptive diagnosis of CGS have been incorrectly diagnosed (author, personal observation). The diagnostic accuracy is likely to be lower in regions with a low incidence of the disease (Doxey et al., 1995a).

The desired aim of most diagnostic tests is to achieve 100% sensitivity and specificity compared to the gold standard. Relating to EGS, the most important diagnostic determinant is specificity, as false positive results may result in unnecessary euthanasia of horses erroneously diagnosed with EGS.

1.6.1 Ancillary diagnostic tests

1.6.1.1 Haematology and biochemistry

Haematology and biochemistry is of limited value in the diagnosis of EGS. Evidence of haemoconcentration (increased PCV, serum protein and serum urea) and stress (increased cortisol) are evident in acute EGS; however these findings are not unique to EGS (Doxey et al., 1991c). Whilst the serum activity of the intestinal isoenzyme of alkaline phosphatase was elevated in acute EGS, the mean proportion of intestinal to total alkaline phosphatase remained within the normal reference range (Doxey et al., 1991c). Ten of 15 EGS horses had a mild metabolic alkalosis (Fintl et al., 2002).

1.6.1.2 Acute phase proteins

Milne et al. (1991) demonstrated an increase in haptoglobin and orosomucoid in all sub-classifications of the disease and in horses with active inflammatory conditions, but not in control and non-inflammatory colic cases. Ceruloplasmin and ∝2-macroglobulin were significantly higher in acute EGS only; thought more likely to reflect haemoconcentration (Milne et al., 1991). More recently, significant increases in serum amyloid A and fibrinogen were reported in EGS cases compared with healthy horses, co-grazers and non-inflammatory colic cases, but levels were not significantly different from those of inflammatory colic cases (Copas et al., 2013). Whilst fibrinogen and serum amyloid A analysis may have some diagnostic benefits differentiating EGS from non-inflammatory small intestinal obstructions, these tests have a low specificity differentiating EGS from other inflammatory abdominal conditions such as peritonitis, enteritis, colitis and abscessation (Copas et al., 2013).

1.6.1.3 Biomarkers of neurodegeneration

Plasma concentrations of the heavily phosphorylated form of major neurofilament subunit NF-H mirror the degree of axonal degeneration in some human (Petzold and Shaw, 2007) and animal (Shaw et al., 2005) neurodegenerative disorders. However, Stratford et al. (2013) did not detect a significant difference between the levels of this biomarker in plasma from acute EGS and control horses.

1.6.1.4 Cytotoxicity testing

The effects of EGS serum on neuro-2a and genetically engineered PC12 Tet-Off P53 cell lines in an *in vitro* model have been investigated. Serum from 60% of EGS cases exerted cytotoxic effects, such as mitochondrial dysfunction, pinocytosis, and reduced intracellular ATP-content. The low sensitivity, and impracticality of the equipment and time required, questioned the applicability of this ante-mortem, *in vitro*, diagnostic test in practice (Malekinejad et al., 2012).

1.6.1.5 Urinalysis

Whilst urinalysis demonstrated some significant differences between EGS and control horses which may support a diagnosis of EGS, none of the findings were pathognomonic for the disease (Fintl et al., 2002). Horses with EGS had higher urinary specific gravity, creatinine concentration (indicative of haemoconcentration) and protein. Urine pH was significantly lower in EGS cases compared to control and co-grazers, and urine glucose concentration was significantly increased in acute EGS compared to that of subacute EGS and control and co-grazing horses (Fintl et al., 2002).

1.6.1.6 Faecal analysis

The potential diagnostic value of detecting *Clostridium perfringens* in the faeces of EGS cases has been assessed. Detection of *C. perfringens* by ELISA in faecal samples has a good specificity (93%) but poor sensitivity (41%) for differentiating EGS and colic cases (Waggett et al., 2010b).

1.6.1.7 Peritoneal fluid analysis

Peritoneal fluid analytes have been compared in medical and surgical colic, acute and subacute EGS, and control cases. Equine grass sickness cases had a higher specific gravity and protein content than the cases of medical colic. The presence of serosanguinous fluid and high alkaline phosphatase was unique to surgical colic cases and differentiated those cases from EGS (Milne et al., 1990).

1.6.1.8 Phenylephrine eye drops

Resting tone of the upper eyelid is supplied by Müller's superior tarsal muscle, a smooth muscle innervated by sympathetic axons, with second order neurons in the CCG (Hahn and Mayhew, 2000b). Bilateral ptosis is a recognised feature of EGS (Pirie et al., 2014). Reversal of ptosis following the administration of 0.5% phenylephrine (α_1 agonist) to the conjunctival sac confirms the presence of smooth muscle paralysis underlying the ptosis (differentiating from somatic nerve dysfunction), providing evidence to support sympathetic dysfunction in suspected cases of EGS (Hahn and Mayhew, 2000a). However, false positives do occur, especially in sedated horses.

1.6.1.9 Electromyography

As alluded to earlier, EGS results in chromatolysis of many LMN (Hahn et al., 2001). Wijnberg et al. (2006) demonstrated some evidence of neuropathy of skeletal muscles in horses suspected to have EGS by electromyography, and proposed that the combination of clinical and electrophysiological evidence may aid the diagnosis of neurogenic disease in cases of weight loss and colic.

1.6.1.10 Oesophageal motility

In addition to oral and pharyngeal components, it has been proposed that the dysphagia observed in cases of EGS is attributed in part to oesophageal dysfunction (Cottrell et al., 1999). Oesophageal dysfunction was demonstrated by a barium swallow test in all 18 confirmed EGS cases (Greet and Whitwell, 1986); thereby providing supportive evidence for the diagnosis.

Whilst numerous ancillary diagnostic tests for EGS have been evaluated, some tests provide no diagnostic valve, some provide supporting evidence for the diagnosis of EGS, but none have, or even approach, 100% specificity or sensitivity. Furthermore, the use of certain diagnostic tests is impractical in a clinical setting.

1.6.2 Histopathological examination of ileum

At present, a definitive ante-mortem diagnosis is only made following histopathological examination of enteric ganglia (Obel, 1955, Barlow, 1969, Pogson et al., 1992, Doxey et al., 1992, Scholes et al., 1993b, Doxey et al., 1995b). Strangulating and non-strangulating small intestinal obstructions comprise the principal differential diagnoses for acute EGS (Lyle and Pirie, 2009) and in addition to facilitating the collection of an ileal biopsy, surgical exploration of the abdomen in such cases permits the exclusion of such lesions as diagnostic candidates.

Whilst the accuracy of diagnosis of acute EGS has not been reported, in the majority of cases, a clinician experienced in the diagnosis of EGS can differentiate an acute case of EGS from its principal diagnosis, namely physical obstruction of the small intestine. However, if clinical findings are equivocal, an exploratory laparotomy is recommended to provide owners with further definitive confirmation of diagnosis, and, where appropriate, to provide a definitive diagnosis to support an insurance claim for mortality. In the absence of a physical small intestinal obstruction, many horses are euthanased prior to recovery from anaesthesia, before the results of ileal histopathology are available (author, personal observations). In such cases, results of histopathological examination can at best only support the diagnosis retrospectively.

1.6.2.1 Haematoxylin and eosin stained formalin fixed sections of ileum

Examination of haematoxylin and eosin (H&E) stained, formalin fixed full thickness ileal biopsies, has long been considered the best method for ante-mortem diagnosis of EGS (Scholes et al., 1993a, Scholes et al., 1993b, Doxey et al., 1995b, Murray et al., 1997). Critical evaluation by Milne et al. (2010) found that 1cm long, full thickness ileal biopsies, collected post-mortem from 23 cases of EGS and 11 of colic, yielded a sensitivity and specificity of 100% for the diagnosis of EGS, when compared against CCG histopathology as the gold standard diagnostic test.

1.6.2.2 Haematoxylin and eosin stained frozen sections of ileum

Histopathological assessment of frozen (cryostat) sections rather than formalin-fixed sections has the distinct advantage that the diagnosis may be obtained intraoperatively, abolishing the need to recover the horse from general anaesthesia and continue supportive care until a definitive diagnosis of EGS is confirmed or refuted. Cryostat sections of ileum had a sensitivity of 100% and specificity of only 73% for diagnosis of EGS, meaning that a significant proportion of cases would have been misclassified, resulting in inappropriate euthanasia (Milne et al., 2010). Diagnostic errors may reflect freeze artefacts that can mimic neurodegeneration (Milne et al., 2010).

1.6.2.3 Synaptophysin immunolabelling

Synaptophysin is the major membrane protein of synaptic vesicles and is specifically expressed in neuroendocrine tissue (Thiel, 1993). Synaptophysin immunohistochemistry was used to identify accumulation of synaptophysin in degenerating neuronal perikarya in autonomic ganglion and intestinal samples from 6 EGS cases, compared with samples from 4 control horses (Hilbe et al., 2005). Whilst the neuronal perikarya from healthy horses were mainly negative for synaptophysin, a few perikarya showed homogenous faint labelling, and labelling was present around ganglion cells and in the axons of control cases (Hilbe et al., 2005).

Waggett et al. (2010a) further evaluated synaptophysin immunolabelling of ileal sections for EGS diagnosis using a larger sample size (20 EGS and 24 control cases) and including a control group of colic cases. Whilst most samples from EGS cases with identifiable submucosal and myenteric plexus neurons had marked synaptophysin labelling, complete differentiation of EGS cases from control cases was only possible when including assessment of neuronal density and utilising a decision tree (Waggett et al., 2010a).

1.6.3 Alternative biopsy locations

Performing a laparotomy in a suspected CGS case to confirm the diagnosis invariably compromises the likelihood of survival (Doxey et al., 1995a, Doxey et al., 1998) and increases costs. A similar deterioration occurs in cats with dysautonomia which

undergo laparotomy (Milne et al., 1994). Despite this concern, some veterinary hospitals will perform midline laparotomies (Mellor et al., 2013) or standing flank laparotomies to confirm the diagnosis of CGS.

In suspected cases, particularly subacute and chronic cases, where exploratory laparotomy is neither feasible nor clinically indicated, consideration should be given to other appropriate biopsy sites, histopathological examination of which might permit accurate diagnosis.

1.6.3.1 Immunohistochemistry of nasal mucosa

Prince et al. (2003) investigated the ante-mortem diagnostic value of measuring the expression of nonadrenergic noncholinergic neurotransmitters as neuronal markers in nasal mucosal samples from EGS cases. The density of innervation was reduced for all four neuronal markers in chronic cases, compared to acute cases and control horses. The changes in innervation determined in this study may reflect the mechanisms underlying the clinical presentation of rhinitis sicca noted in CGS. However as discussed by Pirie et al. (2014) there is less urgency with regards to the diagnosis of chronic cases and simple visual identification of rhinitis sicca is generally considered pathognomonic for EGS (Lyle and Pirie, 2009).

1.6.3.2 Haematoxylin and eosin stained biopsies of gustatory papillae

McGorum et al. (2015a) achieved 100% sensitivity and 98.2% specificity for diagnosing EGS by the presence of chromatolysis in blinded H&E stained post-mortem sections of subgemmal plexi. Whilst samples from 2 horses were collected post-mortem as ‘simulated biopsies’ using uterine biopsy forceps after the placement of an oral gag, further validation is required to determine the utility of this method in the pre-mortem diagnosis of EGS. However, the presence of a mixture of chromatolytic and normal neurons in 1/57 sections from one of the 13 control horses indicates that chromatolysis of subgemmal neurons is not pathognomonic for EGS (McGorum et al., 2015a).

1.6.3.3 Haematoxylin and eosin stained rectal submucosa

In contrast to nasal and tongue biopsies, rectal biopsies are already commonly utilised for other diagnostic reasons (Lindberg et al., 1996), and are known to be safe, straightforward, inexpensive and minimally invasive (Ricketts, 1996). Furthermore, chromatolytic neurons have already been identified in rectal submucosa in EGS horses (Scholes et al., 1993b, Doxey et al., 1995b, Wales and Whitwell, 2006, Mair et al., 2011). Histological examination of full-thickness, H&E stained, post-mortem rectal samples yielded a sensitivity of 71% and specificity of 100% for EGS diagnosis, when the diagnostic criterion was identification of at least three neurons with complete chromatolysis within the submucosal plexus (Wales and Whitwell, 2006). The number of neurons in individual biopsies varied markedly and only a few neurons were observed in any one section (10mm length and 4mm width) in 9 out of 24 horses, the yield of which could not be maximised by sampling from a particular circumferential site (Wales and Whitwell, 2006). Whilst neuronal depletion is reported in the small intestine of horses with EGS (Pogson et al., 1992, Doxey et al., 1992, Scholes et al., 1993b, Doxey et al., 1995b), a low neuronal count in the submucosal plexus of rectal samples did not appear to correlate with EGS diagnosis, but more likely reflected general paucity of neurons in the rectal submucosal ganglia (Wales and Whitwell, 2006). Staining rectal sections with cresyl fast violet and methyl green-lyronin did not improve the diagnostic value (Wales and Whitwell, 2006).

A later study, utilising uterine biopsy forceps for the collection of partial thickness, H&E stained samples, revealed a sensitivity of only 21% for EGS diagnosis (Mair et al., 2011). The high proportion of inconclusive results was primarily due to an inability to identify reliably sufficient numbers of neurons in the samples that contained only the internal ganglia of the submucosal plexus (Mair et al., 2011). In contrast, the post-mortem samples collected by Wales and Whitwell (2006) were full thickness, and thus contained both the internal and external ganglia of the submucosal plexus. Diagnosis may also be potentially limited by difficulty in histological recognition of chromatolytic neurons and crush artefacts. It was suggested that application of specific neuronal labelling may increase the diagnostic sensitivity of rectal biopsies in EGS diagnosis (Mair et al., 2011).

1.7 Treatment of equine grass sickness

With appropriate supportive nursing care a proportion of CGS cases survive (Milne et al., 1994, Doxey et al., 1995a, Doxey et al., 1998, Doxey et al., 1999, Wylie et al., 2011). Criteria that have been applied to select cases suitable for treatment include; some appetite, an ability to drink and swallow feed and absence of continuous colic signs (Doxey et al., 1995c). One of the greatest obstacles to a favourable outcome is the profound inappetance (Pirie et al., 2014) and cases should be regularly offered a wide variety of highly palatable concentrate feeds of varying consistencies, as food preferences change frequently (Doxey et al., 1995c). Bolus and continuous-flow enteral feeding together with partial and total parenteral nutrition have been used in a few selected cases (Pirie and Jago, 2015). Whilst this may reduce the degree of weight loss and provide a period of time for spontaneous improvement in appetite and reduction in dysphagia, usually the requirement for such nutritional support reflects the severity of the disease and there is currently insufficient evidence to suggest that these forms of nutritional support alter case outcome (Pirie et al., 2014). Both enteral and parenteral nutrition are expensive and associated with multiple potential complications (Pirie and Jago, 2015). In cases that are not too weak, daily walking or pasture turnout can help improve demeanour, appetite and dislodgement of rhinitis sicca plaques (Pirie et al., 2014).

As some morphologically normal neurons remain in the myenteric plexus of the GIT in CGS (Scholes et al., 1993b, Doxey et al., 1995b) it was speculated that the prokinetic agent cisapride might be of therapeutic benefit (Milne et al., 1996). While cisapride increased the rate of passage of digesta and dry matter intake in cases of CGS (Milne et al., 1996), whether this improved survival rate was not determined. This drug is no longer available for the treatment of horses. Brotizolam (an appetite stimulant), acetylcysteine (an antioxidant and neuroprotectant) and aloe vera gel (a plant extract with antioxidant and anti-inflammatory properties) were evaluated as ancillary treatments in 29 cases of CGS; none of the treatments appeared to have any significant benefits (Fintl and McGorum, 2002). Analgesics and hyoscine may be administered to CGS horses to control colic, while omeprazole may be administered to cases that have gastric ulceration and reflux oesophagitis (Pirie et al., 2014). Close monitoring of CGS

cases is also required, to detect reduced faecal output associated with rectal impactions that may require manual rectal evacuation. Close monitoring is also required to detect aspiration pneumonia, a relatively common complication of CGS that can be difficult to detect due to the frequent absence of localising signs, such as a coughing or nasal discharge. Furthermore the associated pyrexia and dullness may be absent because of concurrent treatment with non-steroidal anti-inflammatories drugs (author, personal observation).

1.8 Prognosis

While acute and subacute EGS is invariably fatal, some cases of CGS survive (Milne et al., 1994, Doxey et al., 1995a, Doxey et al., 1998, Doxey et al., 1999, Wylie et al., 2011). The overall survival rate for EGS in a 10 year nationwide surveillance scheme was 16%, and 49% for cases of CGS (Wylie et al., 2011). The survival rate for hospitalised cases of EGS has previously been reported as slightly lower, at 42.7% (Doxey et al., 1995a) and 35.6% (Milne et al., 1994).

Regional variations in survival rate have been reported with cases of CGS in Scotland being more likely to survive (odds ratio 1.7) than those occurring elsewhere in Great Britain; speculated to be due to variation in expertise of care, application of appropriate case selection criteria and/ or disease severity (Wylie et al., 2011). This regional difference in survival rate may also be due to regional perceptions for prognosis of return to previous ridden work, because this may influence the decision to euthanise a horse with EGS. A previous study observed ponies to be significantly less likely to survive than Cob types (Milne et al., 1994), whereas the 10 year nationwide surveillance scheme identified no significant associations between survival and signalment (Wylie et al., 2011). Doxey et al. (1998) also found that Cobs, together with Thoroughbreds, had a higher survival rate, while age and sex had no significant effect on survival. However, this study comprised only 31 recovered cases. Wylie et al. (2011) reported that neither the category of EGS nor the outcome of CGS was significantly associated with age, gender, breed, season or year of diagnosis; except that cases of CGS diagnosed in June were more likely to survive than those diagnosed in May.

As previously alluded to, since the extent of neuronal damage is of a continuum scale, the clinical sub-classifications should also be thought of as such. This is not only applicable to the three sub-classifications but also to the surviving and non-surviving CGS cases. It may be relatively easy to predict the outcome of chronic cases that fall at either end of this continuum of severity. For example, a severe case that is completely anorexic, with severe dysphagia, weakness and inability to stand, is unlikely to survive while a mild case with only a slight reduction in appetite is likely to survive. In contrast, it is more difficult to predict the outcome of cases in the middle of the continuum of disease severity. Moreover, cases of CGS rarely die, being typically subjected to euthanasia hopefully before their welfare is compromised. Ideally, euthanasia should be done before these cases are recumbent and unable to stand, but the time at which this may occur is difficult to predict. Some cases are subjected to euthanasia a few months after CGS has been diagnosed (Doxey et al., 1995a); this is expensive and emotional for owners. Accurate prediction of outcome would have welfare and economic benefits because it could reduce the number of potential survivors (S) which are subjected to euthanasia and the number of non-survivors (NS) which are subjected to predictably fruitless attempts at intensive nursing prior to euthanasia.

Only one previous study has investigated potential clinical factors relating to survival of CGS. It was demonstrated that non-survival was associated with a higher clinical score of dysphagia, anorexia, colic and reduction in intestinal sounds (Milne et al., 1994); clinical signs which may be associated with the degree of neuronal damage. Whilst there was no significant difference between the average rhinitis scores of the two groups, 10 of the 29 NS, but none of the 16 S, had severe rhinitis. No significant differences were identified between S and NS with regards to sweating and muscle fasciculations (Milne et al., 1994). These indices are subjective however and currently no objective criteria for predicting the outcome of CGS cases is available.

In an attempt to predict how long a surviving case of CGS takes to return to clinical normality, using duration of hospitalisation as a proxy for the severity of CGS, Doxey et al. (1999) failed to identify any association between the duration of hospitalisation and the final quality of life of the patient. Multiple factors will affect the duration of hospitalisation, including finances, owner's desire to have the patient return home, and

disease stage at time of admission. Furthermore, in this study, the final quality of life of the patient was subjectively analysed by the owners. However, duration of hospitalisation had a relationship with the return to normal body weight, with horses which had average hospital stays of 8.3 and 45.3 days having an average time for return to normal bodyweight of 4.5 and 6.9 months respectively (Doxey et al., 1999).

1.8.1 Long-term outcome of recovered chronic grass sickness cases

It would be expected that, subsequent to chromatolysis, necrosis and depletion of enteric neurons, the associated dysfunction of the GIT would be irreversible. However, Milne et al. (1994) reported that dysphagia and reduced gut sounds gradually improved in S. Enteric neurons cannot regenerate and it is speculated that a process of compensation, involving the reorganisation of remaining neurons, occurs (Milne et al., 1994).

Horses required between 3 and 18 months before returning to their original weight following hospital discharge (Doxey et al., 1995a) and on average took 12 months before starting ridden work (Doxey et al., 1998). All S in multiple studies resumed ridden work (Milne et al., 1994, Doxey et al., 1995a, Doxey et al., 1998), with up to 81% returning to competition level (Doxey et al., 1995a, Doxey et al., 1998). Despite returning to a normal body weight, some horses continue to have excessive sweating (65%), coat changes (58%), difficulties in swallowing some feed (48%), and mild recurrent colic (23%) (Doxey et al., 1998). The sweating observed in the recovered horses was generally associated with excitement (Doxey et al., 1995a, Doxey et al., 1998). Hair coat changes included abnormal retention of the winter coat and small areas of piloerection, while oesophageal choke was always related to changes in the feeding regimen e.g. introduction of dry fibrous food (Doxey et al., 1995a, Doxey et al., 1998).

A significant loss of enteric neurons was evident in ileal myenteric and submucosal plexuses of horses that recovered from CGS between 11 months to 13 years previously (Doxey et al., 2000, Milne et al., 2005). There was a dramatic reduction in the number of morphologically normal neurons in the ileum of recovered horses (mean number of 0.47 submucosal neurons per cm of circumference in the ileum of recovered horse

compared to 29.4 in healthy control horses), and a less marked reduction in the number of neurons in the jejunum (Doxey et al., 2000).

Whilst there is a reduction in numbers of ICC in the ileum of horses with EGS of all sub-classifications (Hudson et al., 2001), immunohistochemistry revealed a continuous network of ICC in a single recovered case of CGS (Milne et al., 2005). It has been demonstrated that dysfunctional ICC can recover or regain a functional phenotype (Der et al., 2000, Chang et al., 2001). It is speculated that whilst the enteric neurons are permanently depleted, recovered horses no longer demonstrate significant clinical evidence of GIT dysfunction because the ICC recover and compensate for the loss of enteric neurons (Milne et al., 2005).

1.9 Bodyweight loss

Bodyweight loss, potentially resulting from anorexia, dysphagia, rhinitis sicca, loss of taste sensation and cachexia, is a prominent feature of CGS (Hudson and Pirie, 2005, Wylie and Proudman, 2009, Pirie et al., 2014, McGorum et al., 2015a) and is one of the few potentially prognostic clinical parameters that can be measured objectively.

Median weight loss as a percentage of initial hospitalised bodyweight for 34 cases of CGS was 5.2% (including cases that did not lose weight) (Doxey et al., 1995a). Doxey et al. (1995a) reported that those hospitalised CGS cases that lost most weight generally took longer to regain body weight and were more likely to have residual problems, but they were not necessarily more likely to die. However, the NS included in this study (4 out of 34) were those that died at home following discharge from the hospital, and no horses subjected to euthanasia in the hospital were included for comparison. Whilst the body weight loss as a percentage of initial body weight was not compared between S and NS, most likely due to the small number of NS (n = 4), interestingly the horse with the greatest percentage of weight loss (21.5%) was a NS (Doxey et al., 1995a). The median duration of hospitalisation for this study was 31 days, and all horses which lost more than 10% of their body weight were hospitalised for more than 31 days (Doxey et al., 1995a).

1.10 Aims of the studies

The global motivation for the study was to increase the accuracy of diagnosis and prognostication for EGS. An accurate, minimally invasive, ante-mortem diagnostic test for EGS is currently lacking and currently there are no objective criteria for predicting the outcome of CGS cases.

The specific aims of the studies were therefore:

1. a. To determine if submucosal rectal biopsies immunolabelled for β-amyloid precursor protein (β-APP) could provide an accurate ante-mortem test for EGS diagnosis.

   b. To develop and refine a grading scheme for assessing the distribution and intensity of β-APP immunoreactivity within individual neuronal perikarya and axons in rectal submucosal biopsies and subsequently determine the best diagnostic predictor for discriminating EGS and control horses.
2. To determine whether the rate and/ or magnitude of bodyweight change during hospitalisation of CGS cases can provide an objective predictor of survival to discharge from hospital.

2 Chapter Two: Histological assessment of β-amyloid precursor protein immunolabelled rectal biopsies aids diagnosis of equine grass sickness

2.1 Introduction

β-amyloid precursor protein is an extensively post-translationally modified and proteolytically cleaved transmembrane protein, associated with synaptic formation and repair, present at high concentrations within neurons (Muresan and Muresan, 2015). Increased β-APP expression is part of the acute phase response to neuronal injury (Roberts et al., 1994), occurring in acquired diseases (Finnie et al., 2000, Mete et al., 2013), in various neurodegenerative conditions (Hanshaw et al., 2015) including Alzheimer's disease (Salminen et al., 2013), Down's Syndrome (Bandyopadhyay et al., 2013) and tauopathies (Irwin et al., 2013) and in murine neurodegenerative disease models (Kokjohn and Roher, 2009). β-amyloid precursor protein has been shown to accumulate in CCG neuronal perikarya in EGS (McGorum et al., 2015b).

2.2 Hypothesis

This study tested the hypothesis that immunolabelling with β-APP would improve the accuracy of histological assessment of rectal biopsies for EGS diagnosis.

2.3 Materials and methods

Histopathological examination of formalin-fixed, H&E stained CCG is regarded as the "gold standard" diagnostic test for EGS (Doxey et al., 1995b, Pogson et al., 1992, Doxey et al., 1992), and β-APP has already been shown to accumulate in CCG neuronal perikarya in EGS (McGorum et al., 2015b). Histopathological examination of formalin-fixed, H&E stained ileal sections offers 100% sensitivity and specificity

(Milne et al., 2010) and has long been considered the best anatomical site for the ante-mortem diagnosis of EGS (Scholes et al., 1993a, Scholes et al., 1993b, Doxey et al., 1995b, Murray et al., 1997). A grading scheme was initially developed and applied to ileal and CCG samples, to assess the potential sensitivity and specificity of β-APP in EGS diagnosis and to validate the grading scheme, using tissues that yield 100% sensitivity and specificity for EGS diagnosis, prior to its application in rectal biopsies.

2.3.1 Collection of tissue samples

Tissue samples were collected ante-mortem from EGS and control horses for routine clinical diagnostic purposes or, with the horse owners' consent, post-mortem from horses subjected to euthanasia. All ileal (McGorum et al., 2016b), CCG (McGorum et al., 2015b, McGorum et al., 2016b) and some EGS rectal biopsies (Mair et al., 2011) were available from previously reported studies. All the control and remaining EGS rectal biopsies were collected prospectively. Horses were of mixed breeds and gender. EGS was confirmed by post-mortem examination including conventional histopathological examination of H&E stained CCG and/ or ileum (Pogson et al., 1992, Doxey et al., 1992, Doxey et al., 1995b, Milne et al., 2010, Gilmour, 1973, Scholes et al., 1993a), by specialist pathologists experienced in EGS diagnosis. EGS was categorised as previously described (McGorum and Kirk, 2001, Pirie et al., 2014). In summary, acute EGS cases had mild to moderate abdominal pain with gastric and small intestinal distension, subacute EGS cases had less severe signs, a more insidious onset and secondary large intestinal impactions, and chronic cases had none of these sequelae. All control cases with colic had a physical lesion identified at exploratory laparotomy. All other control horses had no ante-mortem clinical evidence of EGS and were subjected to euthanasia for unrelated reasons (see results). Thorough clinical examinations were performed, by clinicians experienced in EGS diagnosis, and specifically there was no evidence of tachycardia, ptosis, muscle fasciculations, patchy sweating, dysphagia, base narrow stance or weight loss with 'tucked up' abdominal silhouette. The reported median age for EGS horses of 5 years (Jago et al., 2015) was considered when selecting cases for control rectal biopsies, such that there would be no significant inter-group difference in age. CCG and full thickness ileal samples were collected post-mortem within 3.5 h of death. Rectal biopsies were collected using

uterine biopsy forceps[a] as described previously (Mair et al., 2011). Post-mortem rectal biopsies were collected within 10 min of death. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and 4μm sections cut. A single section from each rectal biopsy contributed to a single histology slide for each horse.

2.3.2 Immunolabelling methodology

Formalin-fixed, paraffin wax-embedded tissues were dewaxed and rehydrated. Antigen retrieval was performed by microwave heating (96ºC) sections immersed in citrate buffer (pH 6.0, 0.1 M) for 10 min, before cooling for 20 min. A commercial immunolabelling kit[b] was used according to manufacturer's instructions. Slides were rinsed with Tris buffered saline containing 0.5 M Tween pH 7.5 (TBST) and incubated with peroxidase blocking agent[c] for 10 min. Slides were rinsed in TBST and incubated with mouse anti-alzheimer precursor protein antibody[d], at 1:16K for 2 h at room temperature. Method control sections were prepared for all labelled sections, by replacing primary antibody with TBST (for ileum) and with normal mouse serum (for rectal biopsies). Slides were rinsed and incubated with horseradish peroxidase-labelled polymer for 30 min, then rinsed and incubated with substrate chromogen solution[e] for 10 min. Slides were rinsed once in distilled water, counterstained with Harris's haematoxylin for 1 min, dipped in Scott's tap water substitute, dehydrated, cleared using ethanol then xylene and mounted under DPX.

2.3.3 Grading of neuronal and axonal immunolabelling

2.3.3.1 Cranial cervical ganglia and ileum

Initially a grading scheme was developed by assessing the distribution and intensity of immunolabelling of individual neuronal perikarya and axons (Fig 2.1) in randomly selected fields of CCG and ileal sections from a subset of EGS and control horses. The grading scheme was then blindly applied to all sections of CCG and ileum. CCG sections were assessed by grading all individual neurons in five different randomly selected fields (x40 objective) of each section. Ileal sections were assessed by grading all individual neurons in submucosal and myenteric plexi.

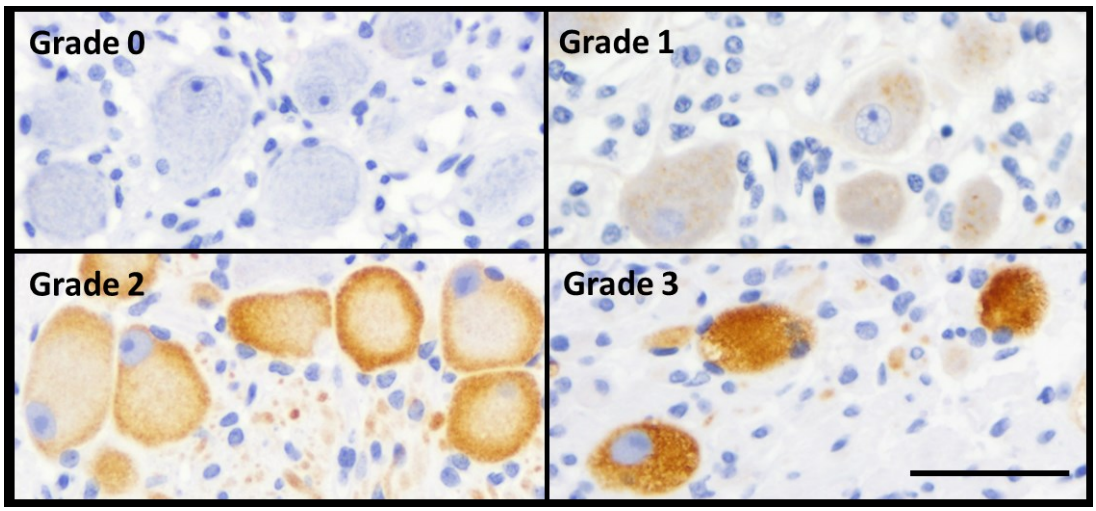


Fig 2.1a: Grading scheme used to assess β-amyloid precursor protein (β-APP) immunolabelling of neuronal perikarya in cranial cervical ganglia (CCG) and ileal sections (bar = 25µm). Grade 0= no labelling; 1= low intensity punctate labelling, but underlying cytoplasm unlabelled; 2= moderate intensity, non-punctate labelling, often appearing as a peripheral halo, but labelling less than 50% of cell; 3= intense labelling of more than 50% of cell.

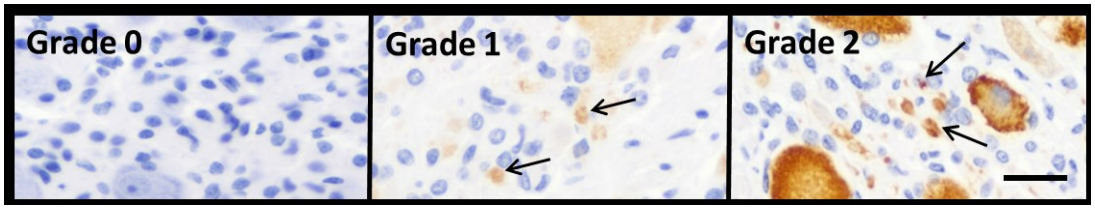
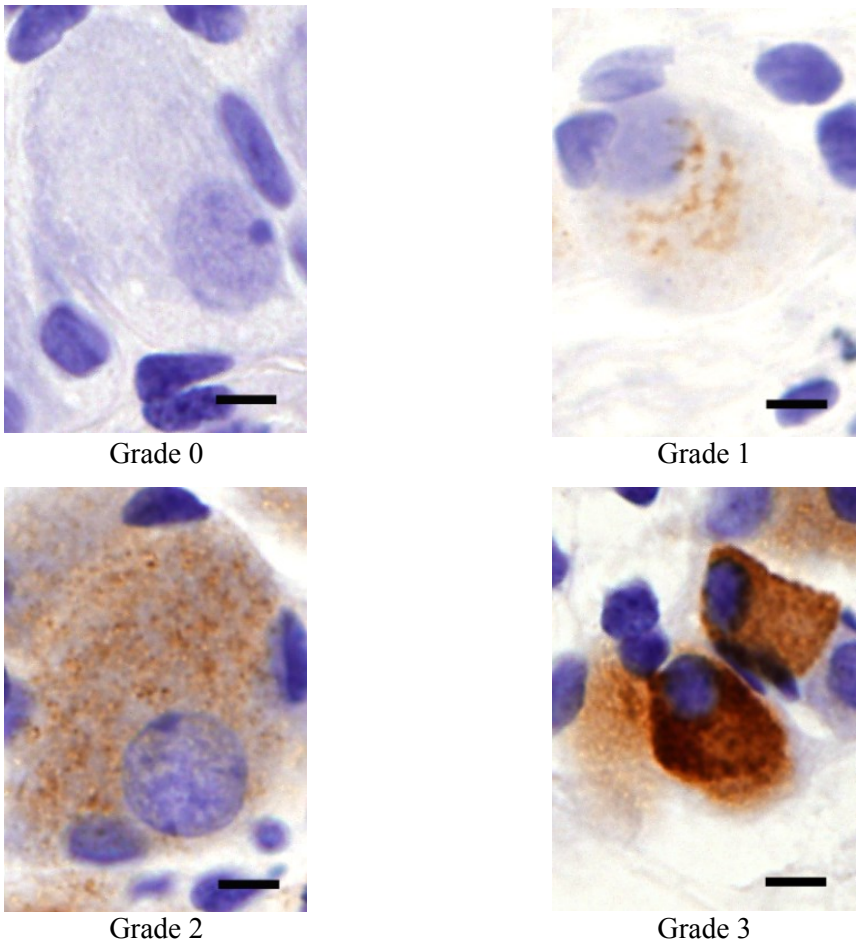


Fig 2.1b: Grading scheme used to assess β-APP immunolabelling of axons in CCG and ileal sections (bar = 10µm). Grade 0= no axon labelling; 1= moderate axon (arrow) labelling; 2= intense axon (arrow) labelling.

2.3.3.2 Rectum

Preliminary screening of rectal samples indicated that the grading scheme developed using the CCG and ileal sections was insufficiently precise to facilitate repeatable and objective grading of the immunolabelling of individual neurons, and in particular to unambiguously differentiate grade 2 and grade 3 neurons. Further refinement of the grading scheme to facilitate objective assessment of the intensity and distribution of immunolabelling of individual neurons was achieved by detailed assessment of the immunolabelling of individual neurons in a randomly selected subset of rectal sections,

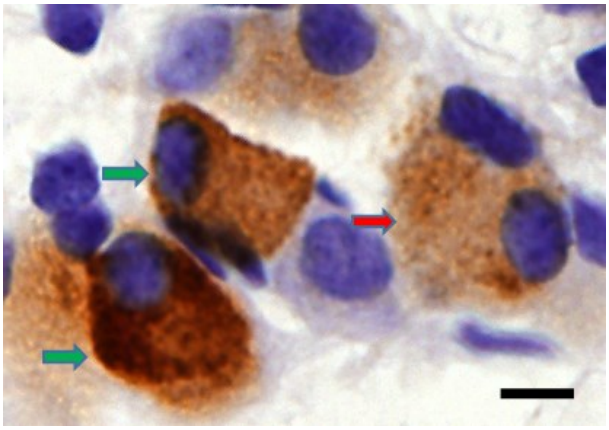
with no regard to disease status. Consequently, the derivation of the neurons (i.e. EGS or control horses) had no bearing on the grading refinement process. When applying the refined grading scheme, grade 3 neurons, in contrast to grade 2 neurons, were classified as those with diffuse labelling of the entire cytoplasm, extending right up to the perikaryonal margin, except when this was displaced by cytoplasmic vacuolation (Fig 2.2 & 2.3). This grading system was then applied by the author in the blinded assessment of all rectal biopsy sections. In all sections, all submucosal plexus neurons containing nuclei were graded, identifying neurons with x10 objective and grading individual neurons with x40 objective.



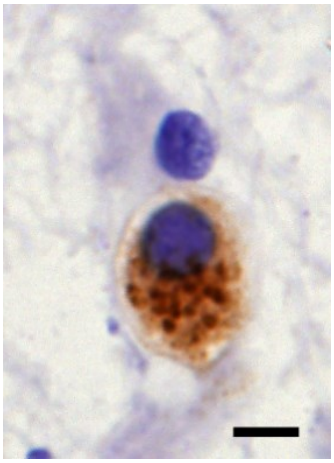
Grade 0 Grade 1

Grade 2 Grade 3

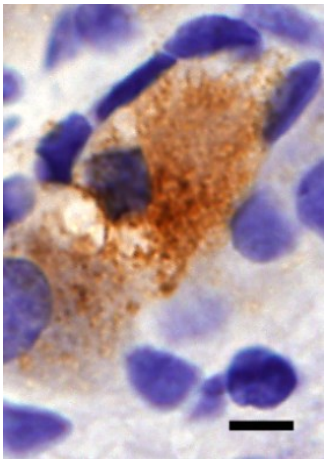
Fig 2.2: Grading scheme used to assess β-amyloid precursor protein immunolabelling of neuronal perikarya in rectal submucosal plexi. Grade 0= no labelling; 1= sparse labelling involving less than half of the cytoplasm; 2= greater than half of the cytoplasm is labelled but areas of unlabelled cytoplasm are still discernible; and 3= diffuse labelling of entire cytoplasm right up to perikaryonal margin with no discernible unlabelled cytoplasm. Bars = 10µm.



2.3a.



2.3b.



2.3c.

Fig 2.3: Examples of potential grading errors (bars = 10µm).

a. Two definitive grade 3 neurons with labelling of the entire cytoplasm right up to the perikaryonal margin and surrounding the nucleus [green arrow], with an adjacent grade 2 neuron with faint basophilic cytoplasm in the background [red arrow]

b. Whilst this neuron does not have labelling extending right up to the perikaryonal margin, this is due to vacuolation of the peripheral cytoplasm. The cytoplasmic membrane itself is labelled and this neuron would be classified grade 3.

c. Grade 3 neuron with peripheral cytoplasmic vacuolation and eccentric and pyknotic nucleus.

2.3.4 Data analysis

Age, number of rectal biopsies per horse and number of neurons counted per horse were described using medians and interquartile ranges (IQR). For each horse, the distribution of different grades of neuron was determined and a weighted immunoreactivity grade calculated. The weighted immunoreactivity grade was the sum of each numerical grade multiplied by its percentage prevalence, and divided by 100. Mann-Whitney U test was used for inter-group (control vs EGS, ante-mortem vs post-mortem sample collection, chronic vs subacute vs acute) comparisons of these variables. Using conventional histopathological examination of H&E stained CCG (Doxey et al., 1992, Doxey et al., 1995b, Pogson et al., 1992, Gilmour, 1973) and ileum (Scholes et al., 1993a, Milne et al., 2010) as the reference tests for EGS diagnosis, the sensitivity and specificity of EGS diagnosis using histological assessment of β-APP immunoreactivity of rectal biopsies was calculated. The predictive value of the distribution of different grades of neurons and of the weighted immunoreactivity grade for EGS diagnosis was determined by constructing receiver operating characteristic (ROC) curves including estimation of area under the curve (AUC). An optimal cut-off was proposed by identifying a point that would give maximum sensitivity with an estimated specificity of 1.0. A specificity of 1.0 was selected (i.e. no false positives within the study data) to minimise the possibility of an erroneously diagnosed EGS horse being inappropriately euthanised. The number of neurons required to be evaluated per horse to be confident that a grade 3 neuron was or was not present, at the expected median prevalence was calculated using a surveillance design tool[f]. The total estimated number of neurons within the rectum required for this calculation was calculated assuming that neuronal density is uniform throughout the length of the rectum (Doxey et al., 1995b), the diameter of submucosal neuronal perikarya was 25μm, and rectal length and diameter was 30 and 7cm respectively (Sisson and Grossman, 1975). GraphPad Prism[g] was used for all other statistical analyses. $P<0.05$ was used as the threshold for statistical significance. This study conformed to Standards for the Reporting of Diagnostic Accuracy (STARD) guidelines where appropriate.

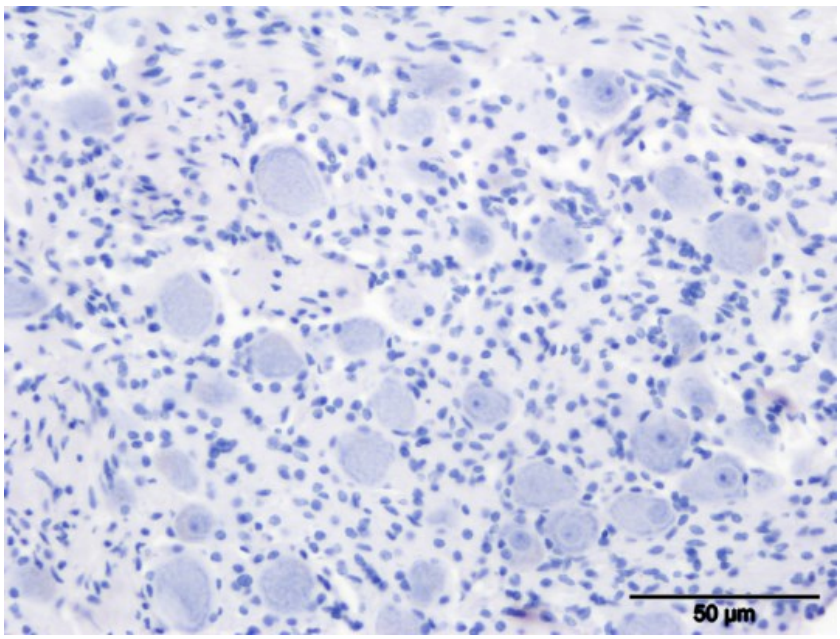
2.4 Results

2.4.1 Cranial cervical ganglion sections

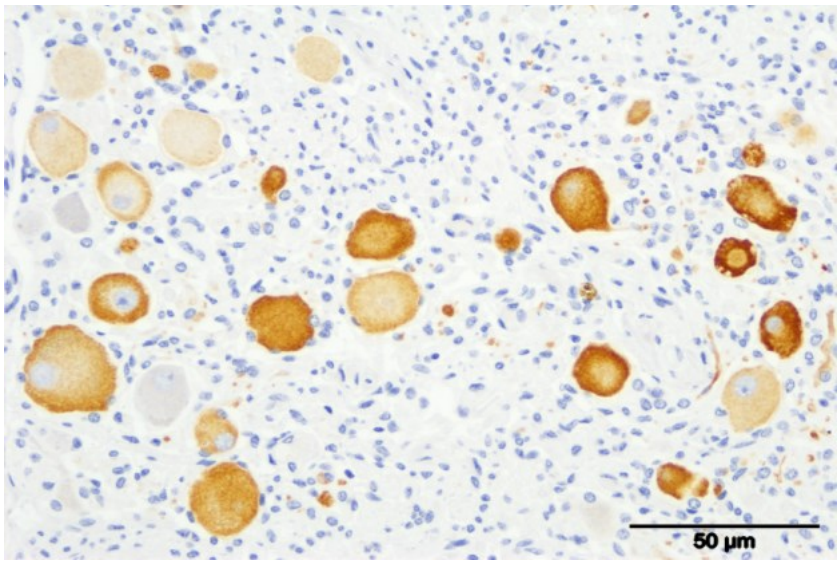
Most neurons from control horses were graded 0-1, limited to low intensity punctate labelling and occasional labelling of adjacent nerve processes and larger diameter nerve fibres, with very little labelling of larger nerve fascicles. Very occasional control neurons were grade 2. In contrast, many perikarya from EGS horses had intense (grade 3) labelling of degenerating neurones and adjacent nerve processes and axons but very little labelling of larger nerve fascicles (Table 2.1 & Fig 2.4). Using the presence of grade 3 neurons as the criterion for EGS diagnosis yielded a sensitivity and specificity of 100%.

Clinical category	Number of neurons	Grade 0 neurons (%)	Grade 1 neurons (%)	Grade 2 neurons (%)	Grade 3 neurons (%)	Axon grade (median)
Control	153	63	37	0	0	0
	147	67	33	0	0	0
	171	47	50	3	0	0
	148	43	56	1	0	0
	169	50	49	1	0	0
Acute grass sickness	183	3	14	42	41	1
	252	2	21	36	41	2
Subacute grass sickness	148	6	30	34	30	1
	218	3	10	69	18	2

Table 2.1: Total number of neurons counted in cranial cervical ganglia sections with grades of β-amyloid precursor protein immunolabelling (% of total neurons) and median grade of axonal labelling.



2.4a.



2.4b.

Fig 2.4: Representative images of β-amyloid precursor protein immunolabelling of cranial cervical ganglia from (a) control horse, and (b) from equine grass sickness horse.

2.4.2 Ileal sections

Differentiation of EGS and control ileal sections was readily achieved because all EGS sections had grade ≥2 submucosal and/or myenteric neurons, while all neurons from control horses were grade <2 (data not shown). Since the presence of grade ≥2 neurons correctly diagnosed EGS with sensitivity and specificity of 100%, further assessment and analysis of the distribution of different grades of individual neurons was not done.

2.4.3 Rectal biopsy sections

Rectal biopsies comprised 21 EGS (6 acute, 12 subacute and 3 chronic) and 23 control horses. Eight EGS samples were collected ante-mortem and the remainder post-mortem. Samples from two control horses were collected ante-mortem and the remainder post-mortem. Reasons for euthanasia for controls collected post-mortem were behavioural (n=1), colic (n=4), neurological (n=4), recurrent uveitis (n=1), orthopaedic (n=7) and elderly horses donated for research (n=4). There was no significant difference in age of control and EGS horses from which rectal biopsies were collected (Fig 2.5).

All rectal biopsy sections comprised mucosa, submucosa with the submucosal plexus, but no myenteric plexus. Significantly more rectal biopsies were collected from controls (median 4; IQR 4-5) than from EGS horses (median 2; IQR 2-2), resulting in a significantly higher total number of neurons counted in control sections (median 187; IQR 111-308) than in EGS sections (median 70; IQR 46-115). However, average number of neurons per section was not significantly different between EGS (median 37: IQR 23-58) and control (median 46; IQR 24-58) horses.

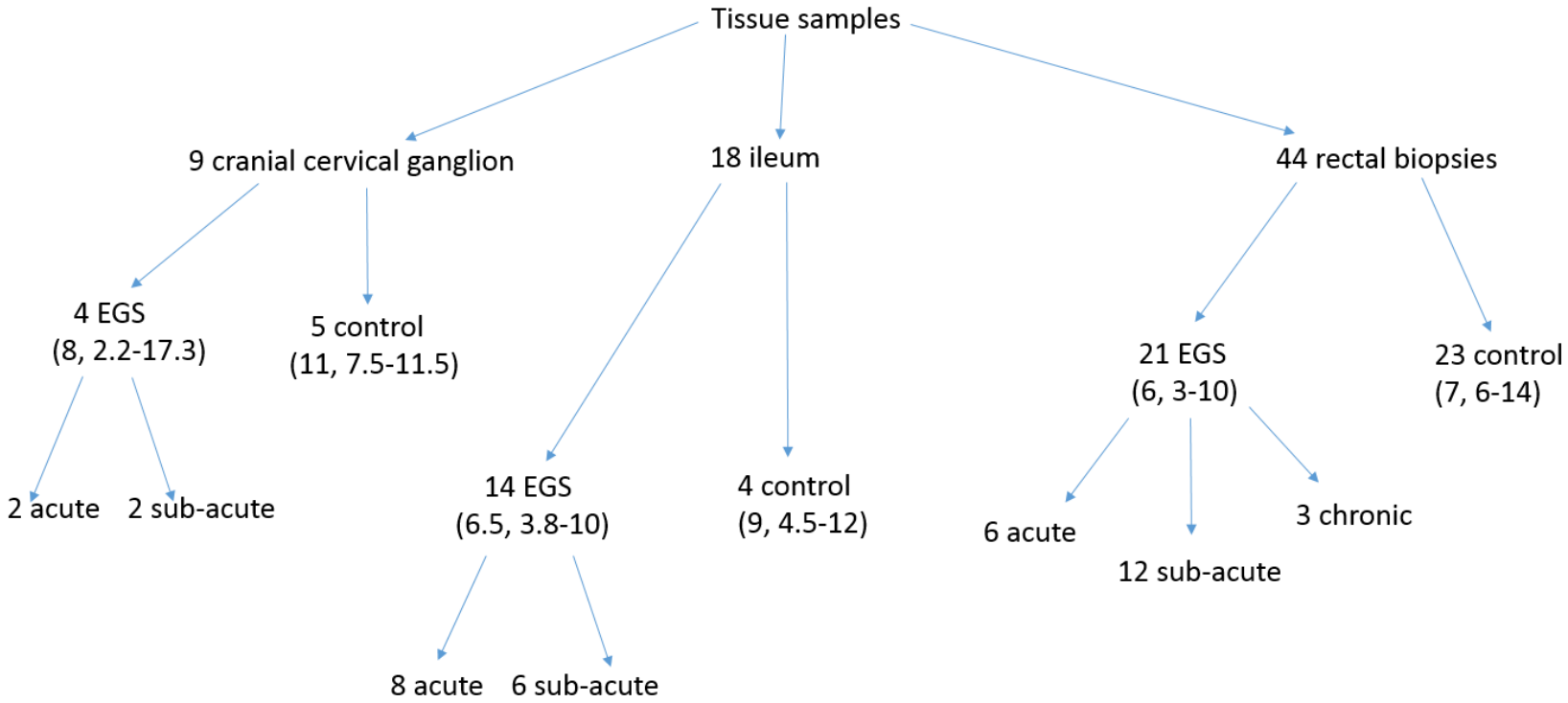


Fig 2.5: Number of samples collected for each clinical category. Median age and interquartile range, in years, are stated in brackets.

EGS = equine grass sickness

EGS horses had significantly lower percentages of grade 0 neurons and significantly higher percentages of grade 1, 2 and 3 neurons (Table 2.2 & 2.3, Fig 2.6 & 2.7). Grade 3 neurons were not observed in control horses, but were observed in all but one EGS case. Very occasional adjacent nerve processes were labelled, but none were detected in the mucosa.

There was no overlap in the weighted immunoreactivity grade for neurons in EGS and control horses, with a value exceeding 1.1 being indicative of EGS. Consequently, the AUC of the ROC curves was highest for weighted immunoreactivity grades, lowest for grade 1 neurons which had the greatest degree of overlap between EGS and control horses, and similar for the other diagnostic determinants (Table 2.3). When the specificity was set at 1.0, the highest diagnostic sensitivity for EGS diagnosis was a weighted immunoreactivity grade exceeding 1.1 (1.0 sensitivity) and a percentage of grade 3 neurons exceeding 0.75% (0.95 sensitivity). Using the presence of one neuron with diffuse labelling of the entire cytoplasm (grade 3) in rectal biopsies as the criterion for EGS diagnosis yielded a 95% sensitivity and 100% specificity for EGS diagnosis.

The weighted immunoreactivity grade for EGS samples collected ante-mortem (median 2.03; IQR 1.46-2.62) was not significantly different (p=0.089) to that for samples collected post-mortem (median 1.58; IQR 1.22-1.84). Further analyses were not performed for control samples, as only 2 were collected ante-mortem. The median number of total neurons per section was higher in chronic cases (median 60; IQR 27-69.5) than acute/ subacute cases (median 34; IQR 23-49); however, the difference was not significant.

The median prevalence of grade 3 neurons in EGS horses was 11.4% (Table 2.3). The total estimated population of submucosal neurons that could be biopsied within the rectum was 23-46,000,000 per horse, depending whether each nucleus was in 1 or 2 adjacent sections. If the total population of submucosal neurons that could be sampled by a rectal biopsy is >5000, grading of at least 30 neurons would be required to have 96% confidence that a grade 3 neuron was or was not present at the expected median prevalence of 11.4% (20 neurons = 89%, 50 neurons = 99.6% confidence).

Clinical category	Grade 0 neurons (%)	Grade 1 neurons (%)	Grade 2 neurons (%)	Grade 3 neurons (%)	Number grade 3 neurons	Weighted immunoreactivity grade	Number of neurons
Control	99.7	0.3	0	0	0	0.003	388
	100	0	0	0	0	0.000	380
	99.4	0.6	0	0	0	0.006	157
	100	0	0	0	0	0.000	341
	100	0	0	0	0	0.000	228
	98.3	1.7	0	0	0	0.017	59
	99.6	0.4	0	0	0	0.004	234
	98.9	1.1	0	0	0	0.011	360
	100	0	0	0	0	0.000	200
	99.6	0.4	0	0	0	0.004	568
	98.7	1.3	0	0	0	0.013	308
	11.3	**71.7**	**17**	**0**	**0**	**1.057**	**53**
	23	**74.3**	**2.7**	**0**	**0**	**0.797**	**187**
	99.1	0.9	0	0	0	0.009	111
	99.3	0.7	0	0	0	0.007	286
	100	0	0	0	0	0.000	121
	91.1	8.9	0	0	0	0.089	123
	85.4	14.6	0	0	0	0.146	158
	76.3	23	0.7	0	0	0.244	139
	69.9	29.2	0.9	0	0	0.310	106
	100	0	0	0	0	0.000	292
	98.4	1.6	0	0	0	0.016	64
	98.2	1.8	0	0	0	0.018	57
Acute grass sickness	1.6	16.1	45.2	37.1	23	2.178	62
	15.7	**37.1**	**47.2**	**0**	**0**	**1.315**	**70**
	4.8	28	41.6	25.6	32	1.880	125
	0	0	8.5	91.5	43	2.915	47
	9.5	50	32.4	8.1	6	1.391	74
	0	5.8	35.5	58.7	71	2.529	121
Sub-acute grass sickness	0	2.1	51.1	46.8	22	2.447	47
	0	0	9.6	90.4	113	2.904	125
	1.2	42.7	46.3	9.8	8	1.647	82
	1.8	**84.5**	**11.9**	**1.8**	**2**	**1.137**	**110**
	5	15	70	10	2	1.850	20
	0	47.7	27.3	25	11	1.773	44
	11.4	27.2	43.2	18.2	8	1.682	44
	3.3	36.7	33.3	26.7	16	1.834	60
	20.5	54.5	13.6	11.4	5	1.159	44
	0	1.2	29.6	69.2	56	2.680	81
	0	**82.1**	**14.3**	**3.6**	**1**	**1.215**	**28**
	14.3	**53.2**	**28.6**	**3.9**	**3**	**1.221**	**77**
Chronic grass sickness	**3.7**	**37**	**57.4**	**1.9**	**1**	**1.575**	**54**
	17.5	**29.1**	**51.7**	**1.7**	**2**	**1.376**	**120**
	6.5	**64.7**	**27.3**	**1.5**	**2**	**1.238**	**139**

Table 2.2**:** Total number of neurons counted in rectal biopsy sections for individual horses and grades of β-amyloid precursor protein immunolabelling (% of total neurons). Data for equivocal cases that had overlapping criteria for the diagnosis of a control or equine grass sickness case are highlighted in red.

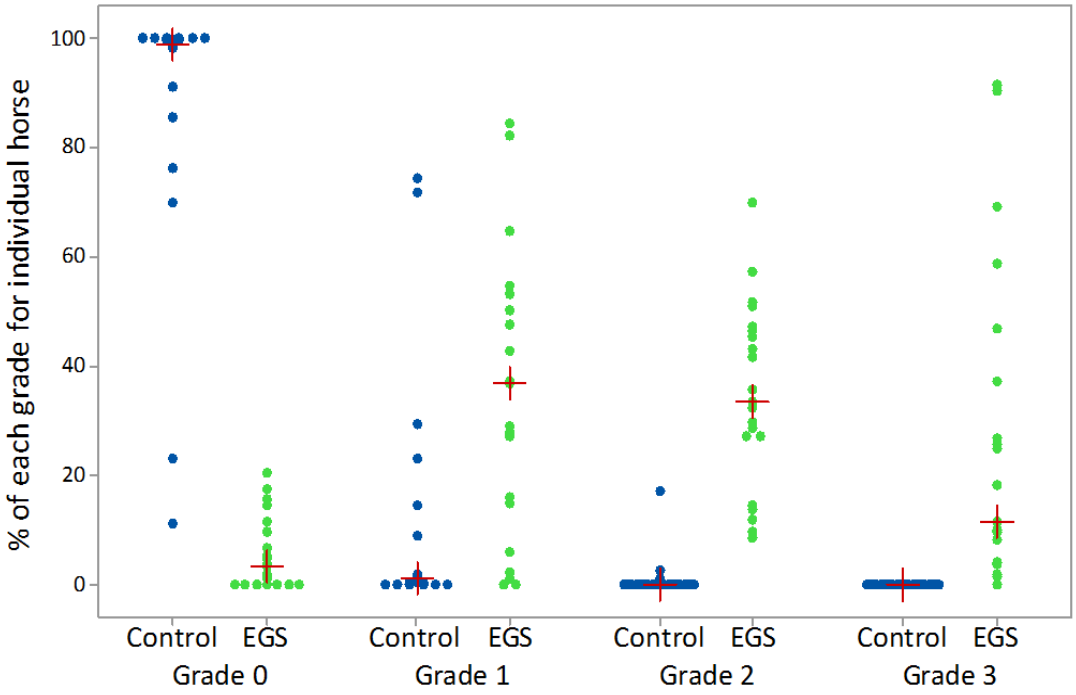
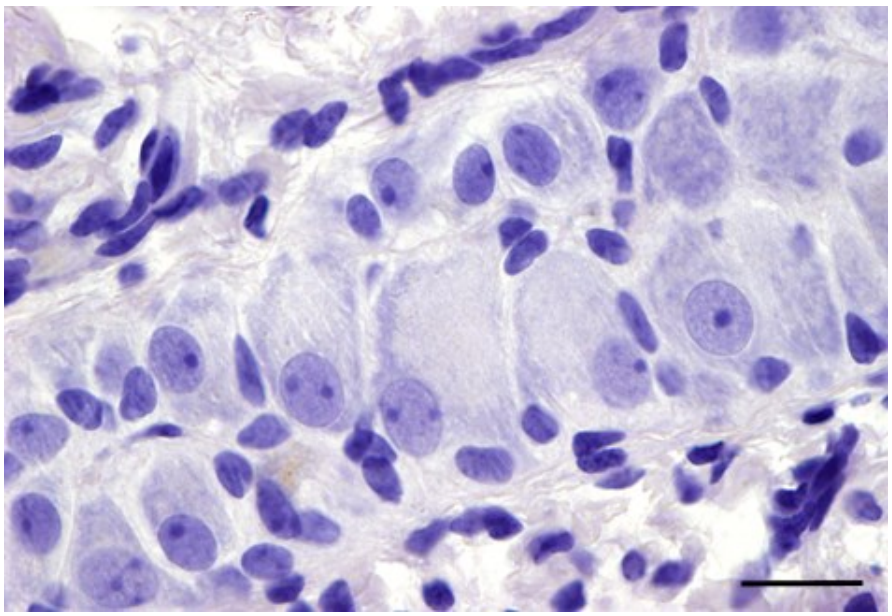
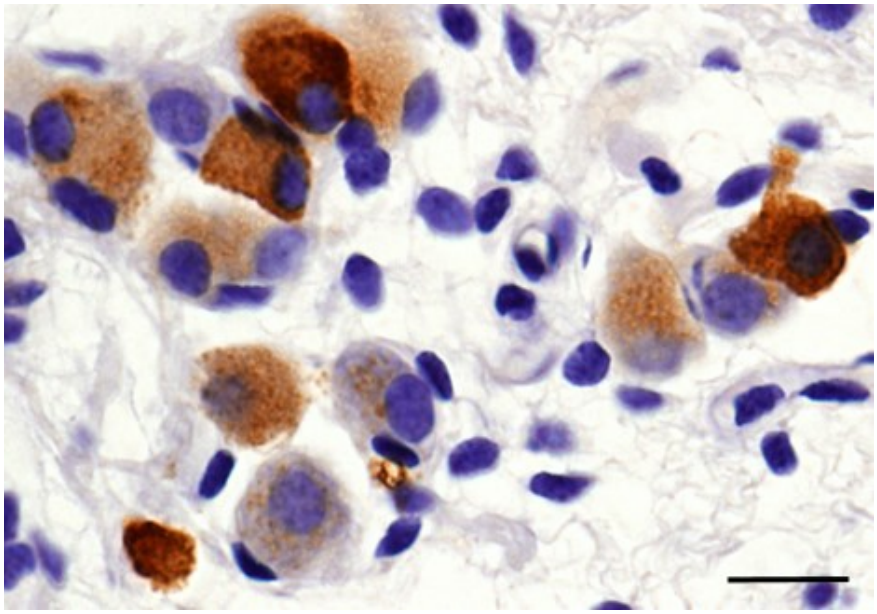


Fig 2.6: Percentage distribution of rectal submucosal neuronal grades for individual horses (red cross = median). EGS = equine grass sickness



2.7a.



2.7b.

Fig 2.7: Representative images of β-amyloid precursor protein immunolabelling of rectal submucosal plexi (a) from control horse, and (b) from equine grass sickness horse (bars = 25μm).

Diagnostic determinant	Control (median & IQR)	EGS (median & IQR)	AUC	95% CI for AUC	Sensitivity (specificity = 1.0)	Threshold for specificity of 1 and highest sensitivity for the diagnosis of EGS
Percentage of grade 0	99.1 (91.1 to 100.0)	3.3 (0.0 to 10.5)	0.99	0.97 to 1.00	0.76	<10.4
Percentage of grade 1	0.9 (0.0 to 8.9)	36.7 (10.4 to 51.6)	0.80	0.66 to 0.94	0.10	>78.2
Percentage of grade 2	0.0 (0.0 to 0.0)	33.3 (20.8 to 46.8)	0.99	0.97 to 1.00	0.76	>22.2
Percentage of grade 3	0.0 (0.0 to 0.0)	11.4 (2.8 to 42.0)	0.98	0.92 to 1.00	0.95	>0.75
Weighted immunoreactivity grade	0.009 (0.0 to 0.09)	1.7 (1.3 to 2.3)	1.00	1.00 to 1.00	1.00	>1.1
Number of grade 3 neurons	0.0 (0.0 to 0.0)	8.0 (2.0 to 27.5)	0.98	0.92 to 1.00	0.95	>0.5

Table 2.3. Diagnostic determinants with associated areas under the receiver operating characteristic curve (AUC), and highest achievable sensitivity, when specificity was set at 1.0, and the thresholds that were set to achieve this, for the diagnosis of EGS.

EGS = equine grass sickness, IQR = interquartile range, CI = confidence interval

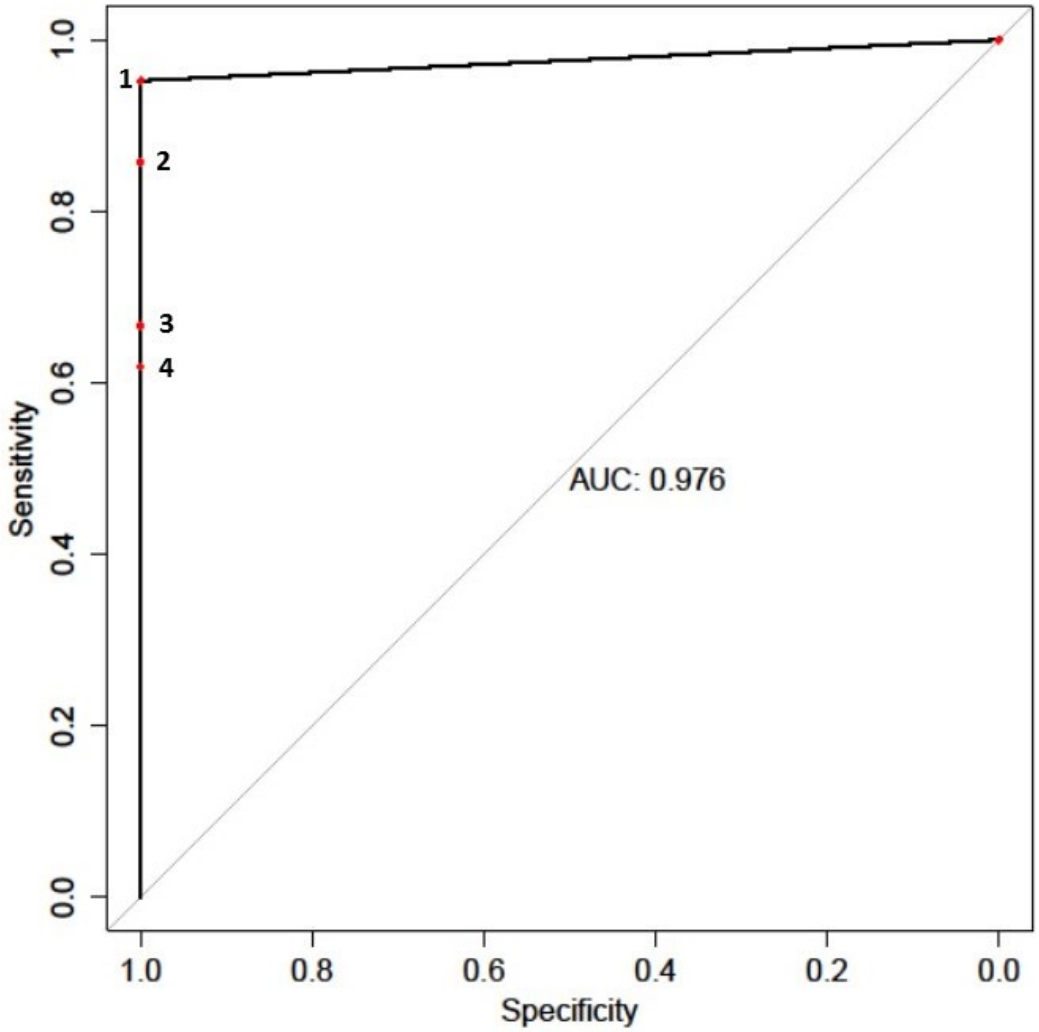
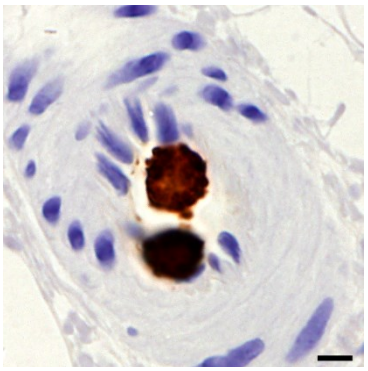


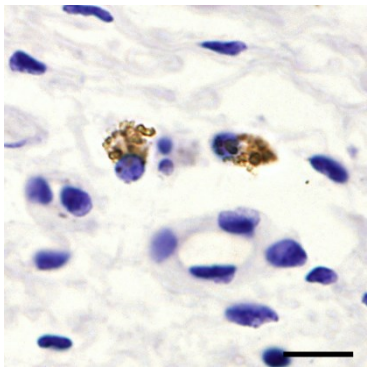
Fig 2.8: Receiver operating characteristic curve for number of grade 3 rectal submucosal neurons required for the diagnosis of equine grass sickness. The numbers adjacent the red dots are the threshold number of grade 3 neurons required for each given sensitivity and specificity.

2.4.4 Non-neuronal labelling and artefacts

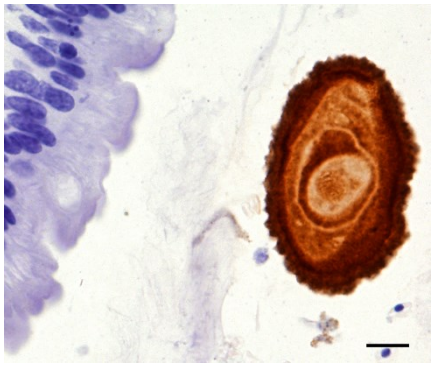
Intimal asteroid bodies (De Oliveira et al., 1985) were unlabelled in the control sections but were immunopositive in other sections (Fig 2.9a). This labelling was readily differentiated from positive neuronal labelling because of their location, protruding into the lumen of small arterioles and lack of nucleus. Pigment granules (haemosiderin and/ or lipofuscin) within macrophages were evident in the submucosa of some horses, and could be distinguished from immunolabelling by the characteristics of the pigment, presence in corresponding control sections and characteristics of cell morphology (Fig 2.9b). Oval particles of plant debris were occasionally translocated from the lumen during the biopsy procedure and could readily be differentiated by morphological characteristics including the absence of nuclei (Fig 2.9c).



2.9a.



2.9b.



2.9c.

Fig 2.9: Examples of non-neuronal labelling and pigment artefacts (a) vascular intimal asteroid bodies (bar = 10µm), (b) macrophages containing intracytoplasmic pigment (bar = 10 µm), and (c) foreign (plant) material adjacent to mucosa (bar = 20µm).

2.5 Discussion

β-amyloid precursor protein immunoreactivity was increased in neuronal perikarya and axons in sections of CCG, ileum and rectum from EGS horses compared with controls. These data extend previous findings of increased β-APP immunoreactivity in CCG sections and increased expression of β-APP in CCG protein extracts in EGS (McGorum et al., 2015b).

β-amyloid precursor protein is synthesised in the endoplasmic reticulum and transported through the Golgi apparatus, plasma membrane and axons (Muresan and Muresan, 2015). The pattern of labelling observed in control rectal submucosal neurons (Fig 2.2: grade 1) is consistent with this normal cellular processing of β-APP, and is similar to that observed in other neuronal populations. In contrast, some neurons in EGS horses had intense granular to diffuse labelling throughout the cytoplasm (Fig 2.3b), indicating abnormal accumulation of β-APP; consistent with dysfunction and degradation of membrane-bound compartments. This study confirms previous work (McGorum et al., 2015b) that EGS is associated with accumulation of β-APP in perikarya of degenerate neurons, in adjacent nerve processes and in intra-ganglionic axons, but not in larger nerve fascicles. Potentially this may reflect (a) upregulation of neuronal synthesis of these proteins, (b) reduced catabolism of β-APP, (c) dysfunction of glycoprotein processing in the Golgi network, and/or (d) failure of axonal transport of protein-containing vesicles to the nerve terminal. Accumulation of β-APP in the perikarya and intra-ganglionic axons, but not in larger nerve fascicles is consistent with the latter. Consistent with the latter two hypotheses, ultrastructural loss of a recognizable Golgi structure is a likely early event in EGS, and EGS is associated with major perturbations in the cytoskeleton of autonomic neurons resulting in accumulation of dopamine-β-hydroxylase and presynaptic proteins in neuronal perikarya (Scholes, 1991, Griffiths et al., 1993, McGorum et al., 2016b).

Evaluation of β-APP immunolabelled rectal biopsy sections using a standardised grading scheme can aid diagnosis of EGS. Indeed, for the sections evaluated in this study, a weighted immunoreactivity grade exceeding 1.1 was 100% specific and sensitive for EGS, and the presence of at least one neuron with diffuse labelling of the entire cytoplasm (grade 3) was 95% sensitive and 100% specific for EGS. This

diagnostic accuracy is comparable to that of conventional histological examination of ileal biopsies (Milne et al., 2010, Scholes et al., 1993a) which has a sensitivity and specificity of 100% (Milne et al., 2010) and is significantly higher than that of conventional histological examination of two rectal biopsies (sensitivity 21%) (Mair et al., 2011). However, it should be stressed that further validation of this technique is required prior to its application to clinical cases.

A subset of CCG, ileal and rectal biopsy samples were pre-screened in order to establish objective grading schemes and to identify non-neuronal staining, prior to blinded evaluation. Individual neuronal perikarya and axons were used as examples of β-APP immunolabelled neurons to facilitate development and refinement of the grading schemes, with absolutely no bearing on whether the neurons were derived from control or EGS sections. The grading scheme was then applied blindly to determine which grading parameter had the greatest diagnostic accuracy in discriminating EGS and control sections.

A limitation of the study was that although the diagnostic criteria facilitated differentiation of EGS and control samples in this sample set, further development and prospective validation of both the grading scheme and the diagnostic criteria using larger numbers of EGS and control samples is necessary before the diagnostic value of this approach can be fully advocated.

While most EGS and control samples could be readily differentiated based on the intensity and distribution of neuronal β-APP immunoreactivity using the criteria of >5% grade 3 neurons (EGS), >40% grade 0 neurons (control) and high proportion of grade 2 neurons (EGS), a sub-population of samples (n=9, highlighted in red in Table 2.2) were considered to be equivocal. Whilst this sub-population would have been correctly classified using the criteria of weighted immunoreactivity grade exceeding 1.1, in a clinical situation, there would be a degree of uncertainty. Consequently, the use of these criteria in a clinical situation would have reduced the confidence in a diagnosis based solely on this diagnostic approach, a significant consideration in light of the fact that a false positive result could prompt euthanasia of a horse without EGS. It is important to note however, that these samples would have been highlighted as equivocal and not erroneously diagnosed. Further work is therefore required to

optimise differentiation of the equivocal sub-population of samples. While a weighted immunoreactivity grade exceeding 1.1 was 100% specific and sensitive for EGS, there are limitations to the use of this criterion for EGS diagnosis. Firstly, there was very little difference between the highest weighted immunoreactivity grade for control horses (1.057) and the lowest weighted immunoreactivity grade for EGS horses (1.137); consequently, it is possible that this criterion may not be fully discriminatory when a larger sample size is prospectively evaluated. Further limitations of this criterion are that it is laborious to determine and likely subject to a degree of inter-observer variability.

Similarly, while the presence of at least one grade 3 neuron was 95% sensitive and 100% specific for EGS, use of this criterion is potentially limited by errors in differentiating grade 2 and grade 3 neurons. To reduce this error, very objective grading criteria were developed and applied, such that grade 3 neurons had diffuse labelling of the entire cytoplasm, extending right up to the perikaryonal margin, except when this was displaced by cytoplasmic vacuolation (Fig 2.3). This potential limitation is confounded by the relative paucity of grade 3 neurons in rectal biopsies of EGS cases (median 11.4% of neurons, IQR 2.8-42) compared to CCG (median 35.5, IQR 21-41). Consequently, EGS diagnosis was based on the presence of only a few grade 3 neurons, and in occasional cases only 1 grade 3 neuron (n=2 horses). Chromatolytic neurons have been reported in the coeliacomesenteric ganglia, jejunum, ileum and small colon in clinically normal horses (Doxey et al., 1995b). Whilst the assessment of chromatolysis is subjective, and relatively objective immunolabelling grading criteria may reduce the likelihood of incorrectly classifying neurons, a larger sample size is required to determine if grade 3 neurons are ever present in control horses. Although increasing the threshold for the diagnosis of EGS to the presence of ≥4 grade 3 neurons would increase the diagnostic certainty, it would also reduce the sensitivity to 62% (Fig 2.8).

Many CCG, ileal and rectal neurons from EGS horses, but not control horses, had pyknotic nuclei (Fig 2.3c). Further study is required to determine whether this conventional histopathological analysis for morphologic features of neurodegeneration, together with chromatolysis and neuronal swelling, could be incorporated into the grading scheme to improve diagnostic accuracy of rectal

biopsies. The assessment of inter- and intra-grader correlation is warranted to determine the repeatability of the proposed grading scheme.

Further work is required to assess the effect of EGS sub-classification on neuronal β-APP immunoreactivity. Consistent with previous work (Scholes et al., 1993b, Doxey et al., 1992), a higher proportion of normal neurons was found in chronic cases; all 3 CGS sections had significantly fewer grade 3 rectal neurons than acute and subacute cases. Consistent with a further study (Doxey et al., 1995b), the median number of total neurons per section was higher in chronic cases than acute/ subacute cases; however the difference was not statistically significant and data from more chronic cases should be assessed to further investigate these findings. Acute and subacute forms of the disease are invariably fatal, while some cases of CGS survive with appropriate nursing (Milne et al., 1994, Doxey et al., 1995a, Doxey et al., 1998, Doxey et al., 1999, Jago et al., 2015). A larger data set of chronic cases is required to investigate the potential prognostic value of β-APP immunolabelling.

Whilst estimations indicate that at least 30 neurons must be examined to be 96% confident that a grade 3 neuron was or was not present, at the expected median prevalence of 11.4%, it is likely that the diagnostic value of rectal biopsies could be improved by increasing the number of biopsies collected from each horse, and by examining multiple non-serial sections cut from individual biopsies. Previous studies indicate that the density of submucosal neurons does not have a consistent circumferential pattern in rectal samples (Wales and Whitwell, 2006), indicating that collection of biopsies from specific locations around the circumference of the rectal wall cannot reliably maximise the number of neurons sampled. While the total number of neurons identified in control rectal biopsies exceeded that of EGS biopsies, this reflected the increased number of biopsies collected from controls. Consequently, there was no intergroup difference in the median number of neurons per section. The median number of rectal biopsies collected for EGS horses was comparable to the previous study by Mair et al. (2011). More biopsies were collected from control horses to increase the confidence of the calculated specificities.

The effect of ante-mortem versus post-mortem sampling must be further evaluated. Both control samples that were collected ante-mortem had lower percentages of grade

0 neurons and were the only control cases that were classified as equivocal. Whilst there was a difference of 0.45 between the median weighted immunoreactivity grades of EGS samples collected ante- and post-mortem, the difference was not significant.

The use of relatively non-invasive rectal biopsies versus ileal biopsies collected at laparotomy to diagnose EGS offers economic, welfare and time benefits. A disadvantage is the increased time required for immunolabelling of rectal biopsies compared with H&E staining of ileal biopsies; total sample fixing and processing times being, respectively, a minimum of 12h and 6h. The timescale may be appropriate for ante-mortem diagnosis of subacute and CGS cases and post-mortem confirmation of EGS in horses that are subjected to euthanasia in the field when the invasive removal of CCG or ileum is not feasible. However, this technique may be unsuitable for rapid ante-mortem diagnosis of acute EGS, unless the time requirement can be reduced, perhaps by using accelerated fixation protocols, frozen sections and employing rapid immunolabelling techniques.

In conclusion, this work has demonstrated the potential diagnostic value of immunolabelled rectal biopsies for EGS diagnosis. However, further prospective studies are required before the use of this technique can be fully advocated in clinical decision making.

2.6 Manufacturers' addresses

[a]Equivet uterine biopsy forceps; Kruuse UK Ltd, Sherburn in Elmet, UK

[b]DakoCytomation EnVision+ System-HRP; DAB K4001; Dako, Ely, UK

[c]Dako Real Peroxidase Blocker S2023; Dako, Ely, UK

[d]MAB, clone 22C11, mouse anti-alzheimer precursor protein A4 antibody; Millipore, Watford, UK

[e]Liquid DAB; ImmPact Dab SK4105; Vector Laboratories, Peterborough, UK

[f]http://epitools.ausvet.com.au

[g]GraphPad Software, La Jolla, California

3 Chapter Three: Bodyweight change aids prediction of survival in chronic equine grass sickness

3.1 Hypothesis

This study tested the hypothesis that magnitude and/ or rate of bodyweight change from first weighing could provide an objective predictor of outcome in CGS cases, with the magnitude and rapidity of bodyweight loss being less in survivors (S).

3.2 Materials and methods

3.2.1 Study design and data collection

A single centre retrospective observational study was conducted. Records of all horses admitted for management of CGS to The Dick Vet Equine Hospital between 1998 and 2013, inclusive, were analysed. Information obtained from records included age (years), gender (female/ entire male/ gelding), breed, survival to hospital discharge; hereafter referred to as survival status (S/ non-survivors (NS)), duration of disease at admission (days) as reported by the owner, duration of hospitalisation (days), body weights (kg), and indication(s) for euthanasia that were recorded in the final clinical report. Survival was defined as discharge from the hospital. Horses were excluded if they met the following case exclusion criteria: <2 weights were recorded or exploratory laparotomy was performed. Body weights were determined using a weighbridge[a] at variable times throughout the hospitalisation period.

3.2.2 Patient management

Horses were nursed according to published guidelines (McGorum et al., 2009), consisting predominantly of offering a variety of highly palatable concentrate feeds of varying consistencies. Initially cases were fed every 2 h, with the frequency reducing as the volume of feed tolerated increased. Horses were walked daily or turned out to grass, provided this would not compromise their weakness. Analgesics and hyoscine

were administered as required for episodes of colic. Some horses also received omeprazole, NSAIDs, antimicrobials, sedatives, diazepam, brotizolam, aloe vera, probiotics, dexamethasone, cisapride, and acetylcysteine. Continuous flow enteral feeding and total or partial parenteral nutrition were used in a few selected cases.

3.2.3 Data analysis

Data were entered into an Excel spreadsheet. Categorical variables were described as percentages and chi-squared tests were used to investigate the association between categorical variables and survival status. As the continuous variables were not normally distributed, they were summarised using medians and IQR. Mann-Whitney U tests were used to investigate their association with survival. Age was analysed as both a continuous and a categorical variable (using categories 1-2, 3, 4, 5, 6, 7, 8, 9-10 and ≥11 years).

Summary statistics were calculated for each horse including minimum weight (as a percentage of first weight recorded), time from first weight recorded to minimum weight, duration of disease on admission and duration of hospitalisation. These were compared between survival status groups using Mann-Whitney U tests. Kaplan-Meier survival and time to discharge curves, from reported onset of disease, were constructed for NS and S, respectively. Individual horse's bodyweights were plotted temporally, as a percentage of the first recorded weight, comparing S and NS.

Referenced to day of first weight recorded, percentage bodyweight changes for each 7 day interval up to 28 days (0-7, 7-14, 14-21, 21-28 days) were used to describe the rapidity of weight loss. Percentage bodyweight changes over entire periods from the first weight (0-7, 0-14, 0-21, 0-28 days) were used to describe the overall magnitude of weight loss. If weight data were not available for the start or end of a 7-day interval, linear interpolation of the weights before and after were used to generate an estimate. Histograms were constructed from these derived data.

The percentage weight changes for all intervals for S and NS were described, and compared using Mann-Whitney U tests. The predictive value of data from each time period for identifying NS was described using ROC curves including estimation of AUC. An optimal cut-off was also proposed by identifying a point that would give

maximum sensitivity with an estimated specificity of 1.0. A specificity of 1.0 was selected (i.e. no false positives within the study data), to minimise the possibility that a potential S would be euthanised inappropriately. Estimated sensitivity was reported together with estimated 95% confidence intervals for sensitivity based on 2000 bootstrap replications of ROC curves.

As a potential clinical discussion tool the percentage weight changes for horses over each interval and period were summarised to estimate the probability of survival of horses grouped into ranges of percentage weight change (percentage survival prediction curves). As these estimates were based on low numbers of individual horses, the uncertainty in the estimates was expressed with binomial exact confidence intervals.

As bodyweights were only recorded during the hospitalisation period and few horses were admitted on the day of onset of clinical signs, weight data were generally not available from the day of disease onset. To determine if correcting for this delay would improve predictive performance, extrapolations were performed to estimate the weight on the day of disease onset i.e. the first day that owners recognised abnormal clinical signs. The extrapolation to day of onset used a quadratic model fitted to each horse's first 6 recorded weights, only when a weight was available $\leq$7 days after the onset of clinical signs, and where $\geq$6 weights had been recorded. These inclusion criteria for extrapolation were selected on iterative examination of fitted values under different criteria and discussion with experienced clinicians blind to the outcome of the individual horse. An extrapolated onset weight was not calculated for horses outside these criteria. Histograms and the associated quantitative data were produced for both extrapolated data (day 0 = day of disease onset) and non-extrapolated data (day 0 = day of first recorded weight) for all time intervals.

The R statistical system[b] was used for all statistical analyses. $P<0.05$ was used as the threshold for statistical significance. This study conformed to Standards for the Reporting of Diagnostic Accuracy (STARD) guidelines where appropriate.

3.3 Results

Since 28 of the 241 horses hospitalised for management of CGS met the case exclusion criteria, the study sample comprised 213 horses. Median age was 5 years (IQR 3.5-8). There were 96 (45.1%) females, 104 (48.8%) geldings and 13 (6.1%) entire males. Breed categories consisted of 22 (10.3%) Scottish native ponies, 31 (14.6%) other ponies, 19 (8.9%) Thoroughbreds, 42 (19.7%) cobs, 17 (8%) draft horses, 11 (5.2%) warmbloods, 13 (6.1%) other pure breeds and 58 (27.2%) crossbreeds. No signalment variables were associated with increased risk of non-survival.

There were 114 (53.5%) S and 99 (46.5%) NS. The survival rate was (53.5%) for the 213 horses included in the study, and 49.4% for all (241) horses hospitalised for management of CGS during the study period. All NS were euthanised, with none dying. For 92 of the 99 NS, information was available describing the indications of euthanasia. The most prevalent (29.3% of NS) single indication for euthanasia was recumbency and inability to stand. Seven cases were euthanised for other single indications including aspiration pneumonia, functional post-renal obstruction, cardiovascular collapse, diarrhoea, persistent anorexia and recurrent colic. Fifty eight horses (63.1%) were euthanised for multiple indications (Table 3.1). Although weight loss was the most prevalent indication when multiple indications for euthanasia were reported, horses were never euthanised solely because of weight loss.

	Frequency	Percentage (of 58 horses)
Weight loss	28	48.3
Anorexia	24	41.4
Progressive weakness	21	36.2
Dysphagia	20	34.5
Deteriorating demeanour	19	32.8
Rhinitis sicca	19	32.8
Deteriorating appetite	16	27.6
Recurrent colic	10	17.2
Diarrhoea	8	13.8
Other	6	10.3
Acute severe colic	5	8.6
Recumbent and unable to stand ($\pm$ assistance)	2	3.4
Gastric reflux	2	3.4
Functional post-renal obstruction	2	3.4
Aspiration pneumonia	2	3.4
Persistent tachycardia	2	3.4

Table 3.1: Frequency of reported indications for euthanasia in 58 horses that had >1 indication for euthanasia.

There was no significant difference in age of S (median 5 years, IQR 3-8) and NS (5 years, 4-8), nor in duration of disease prior to hospitalisation (S 6 days, IQR 2-10; NS 5, 2-8). Survivors were hospitalised for significantly longer than NS (S 34 days, IQR 22-60; NS 14, 10-25; $p<0.00001$) (Fig 3.1). Kaplan-Meier survival curve indicated that 50% of NS were euthanised by 21 days and 75% by 32 days from onset of disease (Fig 3.1a). A time to discharge curve for S indicates that 50% were discharged by day 42 from onset of disease (Fig 3.1b).

Horses were weighed on average every 2 days (IQR 1-3); the frequency was not significantly different between S and NS. Compared with NS, S had significantly lower maximum bodyweight loss as percentage of first weight (S 5.9%, IQR 1.8-13.5; NS 12.7, 6.4-17.3; $p<0.0001$), and a significantly earlier day of minimum weight (S day 17, 13-27; NS 23, 17-33; $p=0.0008$). All NS lost weight, whereas some (45.6%) S gained weight or shortly reached their nadir and then rapidly increased, making the overall gradient of the curve less in S (Fig 3.2). The highest percent of total bodyweight loss by individual S and NS was similar (36% and 37%, respectively).

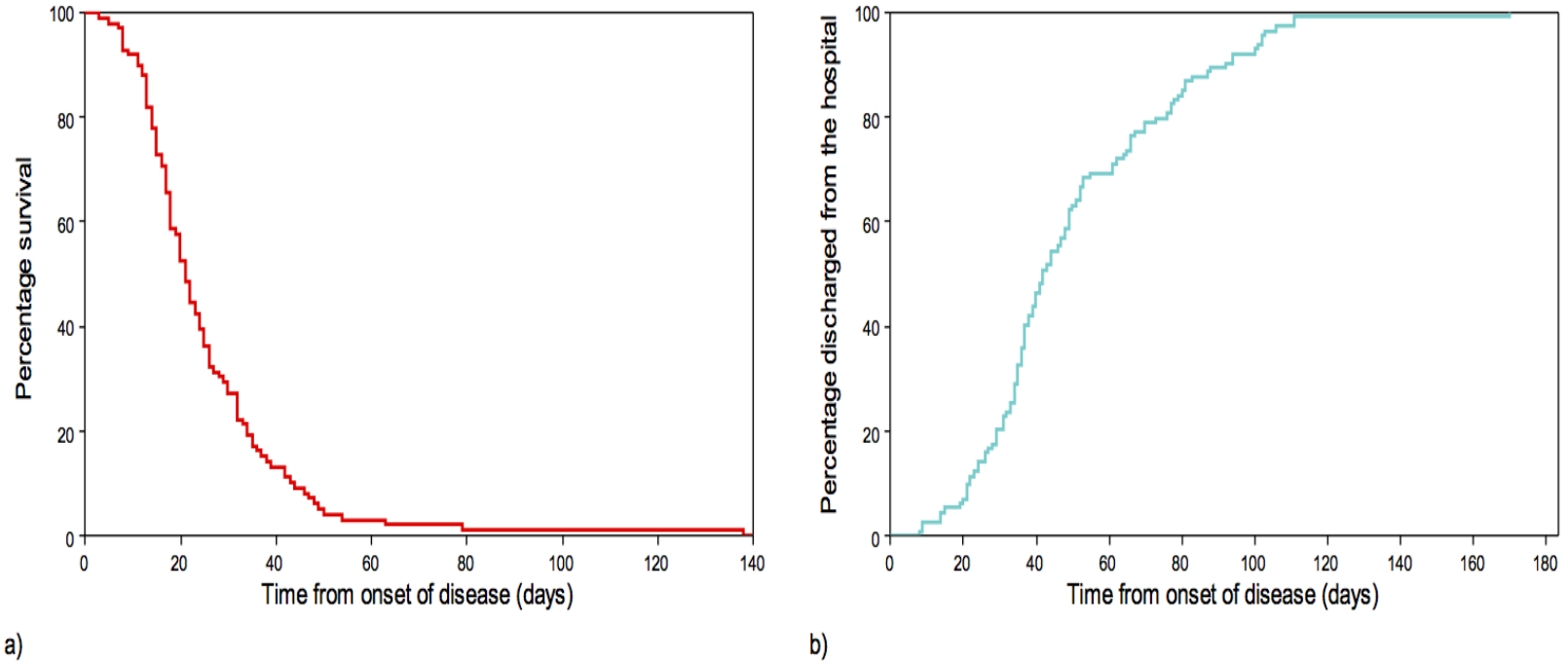


Fig 3.1: (a) Kaplan-Meier survival curve for 99 non-survivors, and (b) curve describing the duration of hospitalisation for 114 survivors.

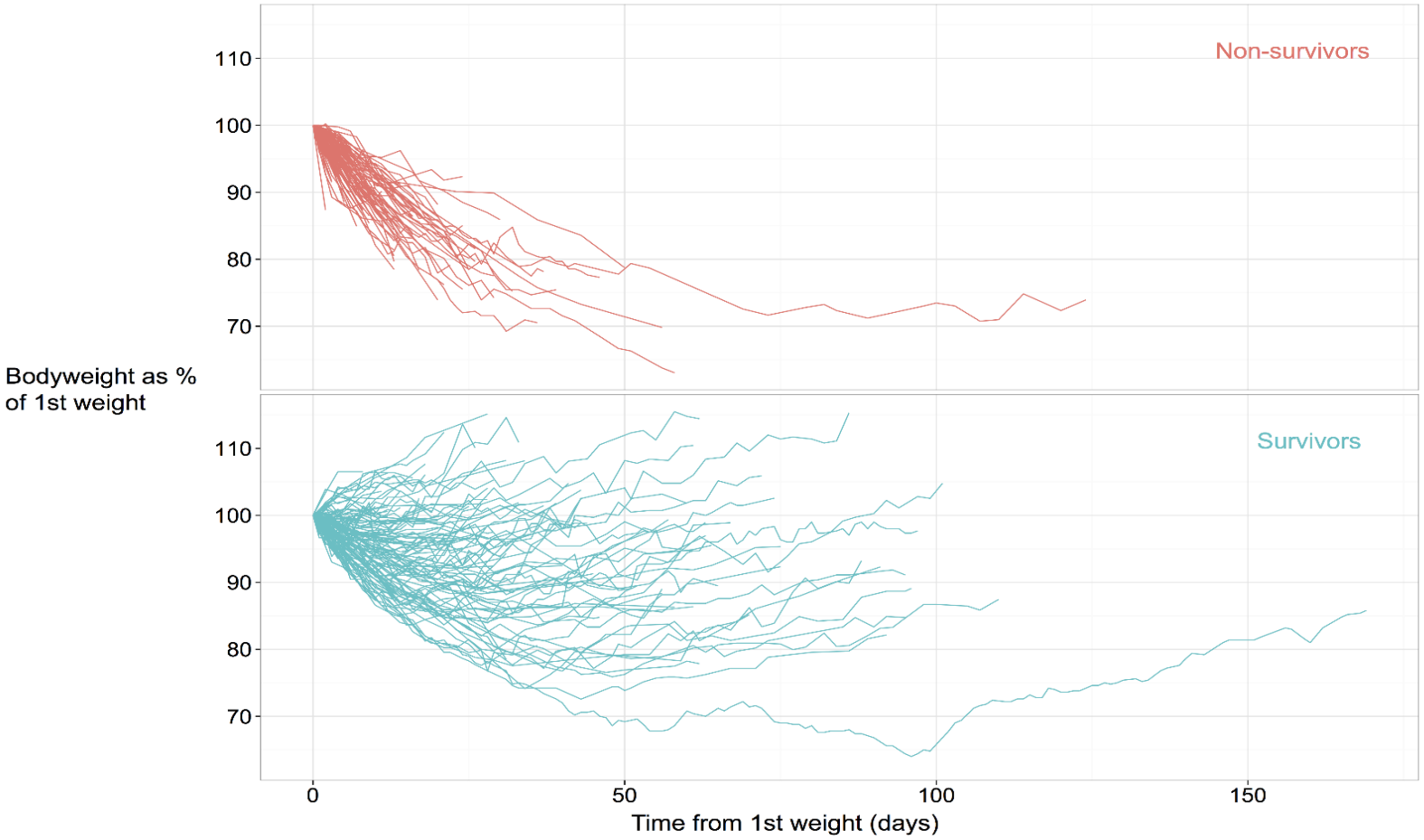


Fig 3.2: Temporal courses of body weights for individual horses as a percentage of the first recorded weight, for non-survivors and survivors.

Time interval (days)	Survivors (median & IQR %)	Non-survivors (median & IQR %)	Difference between the medians of S & NS (p < 0.001 for all)	AUC	95% CI for AUC	Sensitivity (specificity =1.0)	95% CI for sensitivity
0 to 7	-3.4 (-6.4 to -1.1)	-7.2 (-9.6 to -5.5)	3.80	0.82	0.76 to 0.88	0.21	0.12 to 0.32
7 to 14	-1.8 (-4.9 to 0.2)	-5.9 (-7.4 to -4.7)	4.10	0.84	0.77 to 0.91	0.22	0.10 to 0.37
14 to 21	-0.02 (-2.6 to 0.9)	-5.4 (-6.6 to -5.0)	5.38	0.91	0.84 to 0.98	0.09	0.00 to 0.77
21 to 28	-0.2 (-2.3 to 1.7)	-5 (-5.5 to -2.8)	4.80	0.85	0.76 to 0.95	0.08	0.00 to 0.25
0 to 7	-3.4 (-6.4 to -1.1)	-7.2 (-9.6 to -5.5)	3.80	0.82	0.76 to 0.88	0.21	0.12 to 0.32
0 to 14	-6.1 (-10.3 to -0.9)	-12.3 (-15.1 to -10.1)	6.20	0.83	0.76 to 0.90	0.20	0.10 to 0.37
0 to 21	-7.5 (-12.9 to -2.8)	-16.6 (-18.7 to -15.1)	9.10	0.88	0.81 to 0.95	0.18	0.05 to 0.36
0 to 28	-9.2 (-15.5 to -2.2)	-19.9 (-22.8 to -18.1)	10.70	0.87	0.78 to 0.96	0.25	0.08 to 0.50

Table 3.2: Median percentage bodyweight changes for various time intervals for survivors and non-survivors from the first recorded weight, with associated areas under the receiver operating characteristic curve (AUC) and highest achievable sensitivity, when specificity was set at 1.0. IQR = interquartile range, CI = confidence interval.

Using overall magnitude and rapidity of bodyweight change data referenced to first weight, S had significantly lower weight loss than NS at all periods listed in Table 3.2. The greatest percentage of bodyweight loss within a 7 day period occurred between 0 and 7 days, for both S and NS. The AUC was similar for all time periods, but highest between 14 and 21 days. When the cut-off was set to give a specificity of 1.0, sensitivity was fairly low for all time periods, but was greatest for the 7 to 14 day interval (Table 3.2).

The percentage bodyweight change of S and NS from day 0 (first weight) to 7 is presented in Fig 3.3, with histograms for other time intervals being presented in Fig 3.4. Percentage survival prediction curves are shown in Fig 3.5.

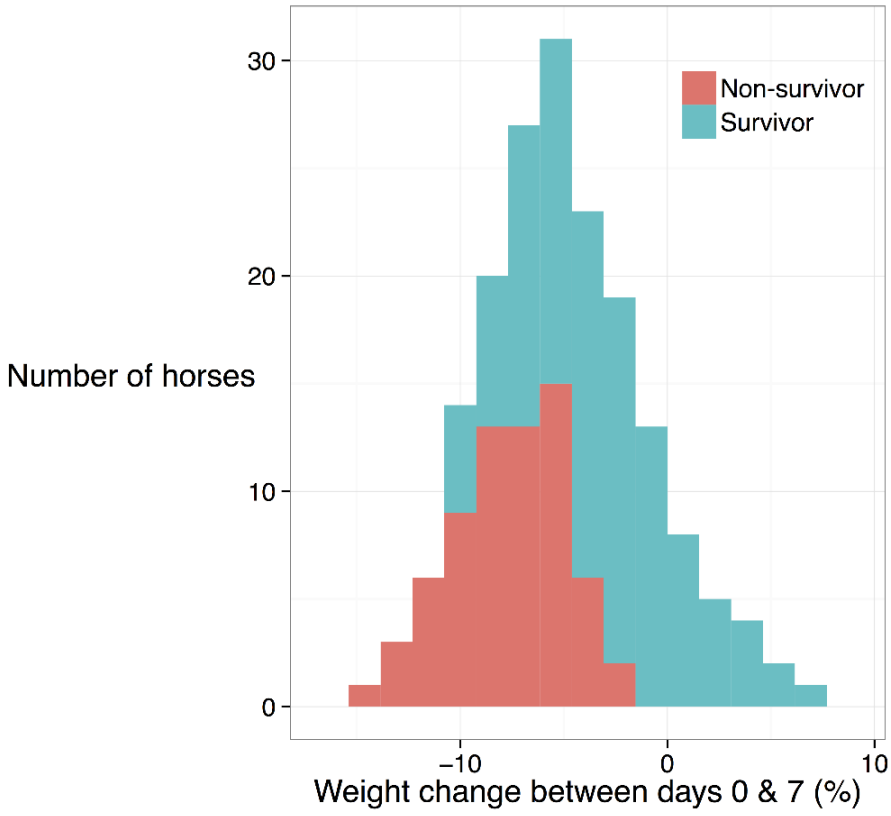


Fig 3.3: Histogram demonstrating the percentage bodyweight change of survivors (S) and non-survivors (NS) from day 0 (1st weight) to day 7. For example, 13 horses lost 2% bodyweight and all these were S. Six horses lost 12% bodyweight and all were NS. Thirty one horses lost 6% bodyweight and these horses were a mixture of S and NS.

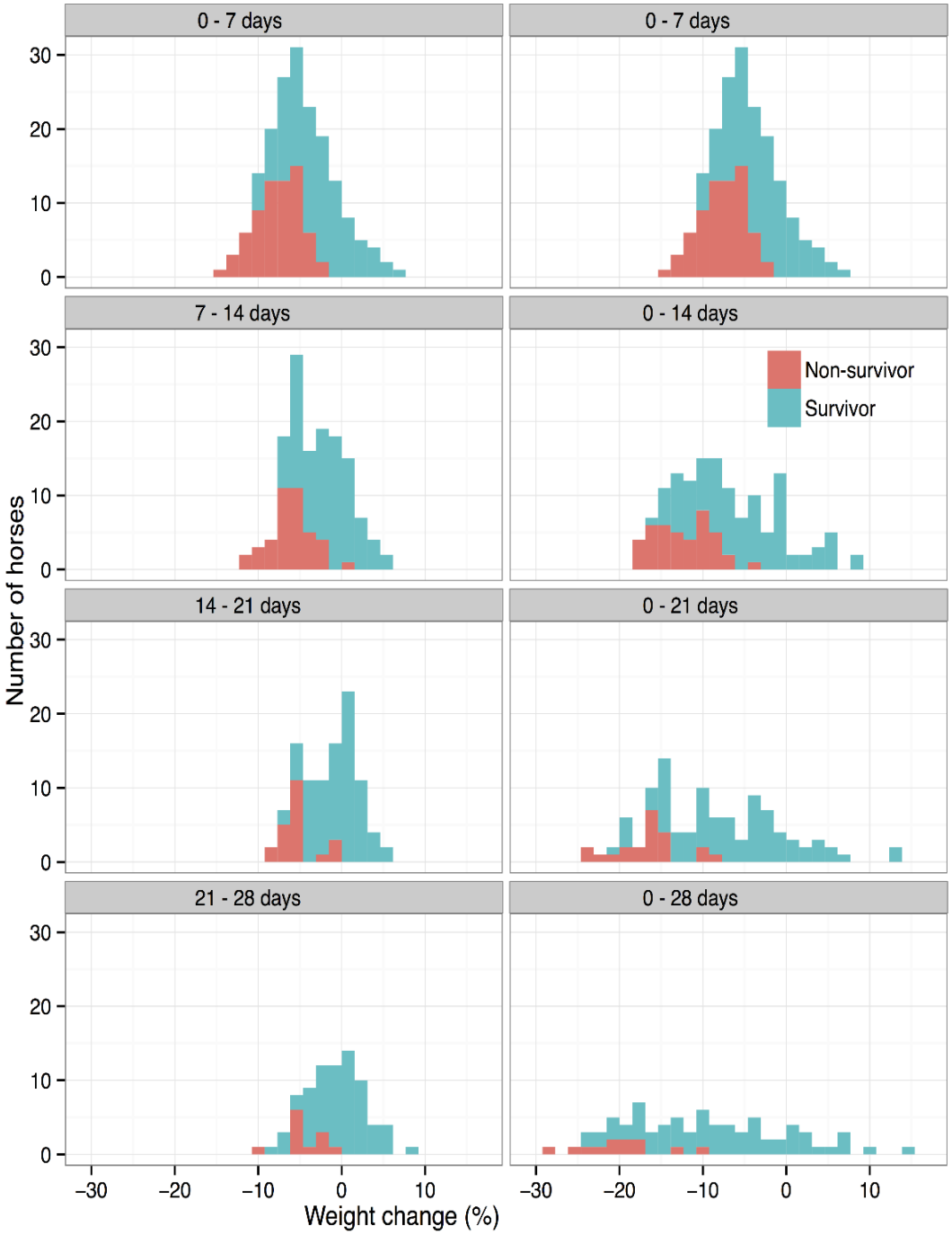


Fig 3.4: Histograms demonstrating the percentage bodyweight change of survivors and non-survivors for each 7 day period and each period from the time of first weight up to 28 days, in 7 day increments.

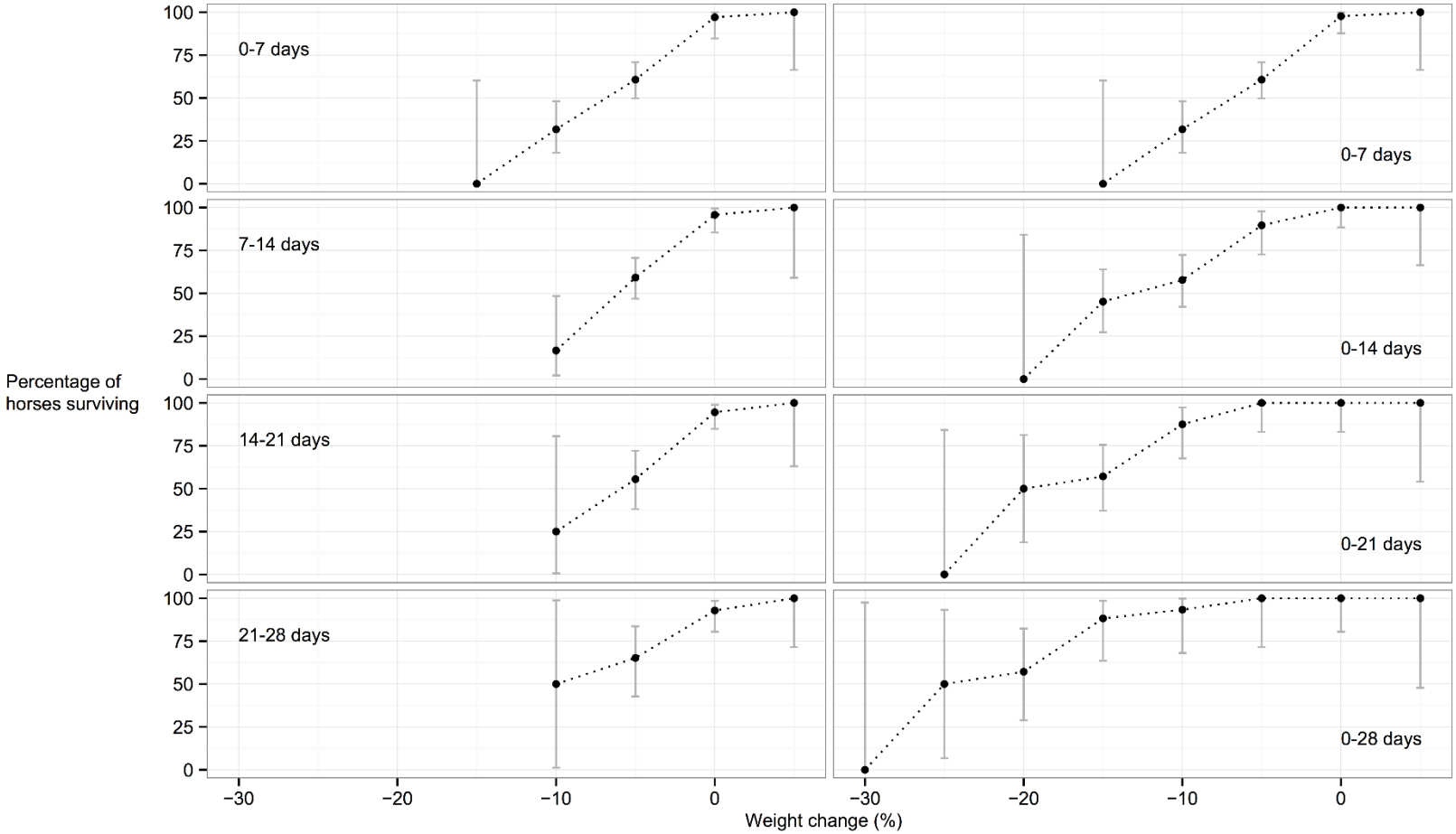


Fig 3.5: Percentage survival prediction curves for each 7 day period and each period from the time of first weight up to 28 days, in 7 day increments.

All the aforementioned results reported and later discussed are based on non-extrapolated data, with day 0 as the first weight recorded. Only 93 horses met the criteria for extrapolation to estimate a weight at onset of disease. The predictive performance measures based on extrapolated weights were overall less informative than the measures based on first weight recorded (AUC lower for 7/8 models based on extrapolated weight compared to first weight). As extrapolation only estimates the weight at onset, it is possible that the standardising of timing it adds is overwhelmed by the error introduced by the quadratic model extrapolation.

3.4 Discussion

This is the largest study to report the outcome of hospitalised CGS cases (Milne et al., 1994, Doxey et al., 1995a, Doxey et al., 1998, Doxey et al., 1999). The survival rates of 49.4% for all (241) horses hospitalised for management of CGS during the study period, and of 53.5% for the 213 horses included in the study, were higher than previously reported (35.6% (Milne et al., 1994) and 42.7% (Doxey et al., 1995a)). The difference in percentage survival of all 241 horses compared with the 213 horses used in the study reflects the higher number of NS in the excluded sample (23/28), likely because some were euthanised soon after admission, prior to recording the $\geq$2 weights required for study inclusion.

For any given change in percentage bodyweight over any of the defined time intervals the percentage survival prediction curves report the survival rate in our study sample gathered over the last 16 years. These curves can be used to predict the survival of future cases. Obviously each clinical case should be assessed on a case by case basis, for example if the horse has concurrent aspiration pneumonia or colic, or is one of the outliers, but these values can be used as a prognostic discussion aid derived from previous outcomes, to help guide owner decisions. For example, if a horse had lost 5% bodyweight after 21days and the owner was considering euthanasia, our data indicate that 100% of horses losing this amount of weight over this time period previously survived to discharge.

Compared with NS, S had significantly lower median maximum bodyweight loss overall and for each individual time period analysed. The greatest difference in median weight loss of S and NS occurred between days 14 and 21. The greatest AUC was also for this time period, however the confidence intervals of all areas overlap, so we cannot report that this value is significantly greater. Whilst the AUC is an objective measure of overall discrimination, both this and the difference between the medians is a population statistic and is not indicative of the best diagnostic predictor for discriminating S from NS on an individual basis. With a guaranteed specificity of 1.0, the highest achievable sensitivity was 0.22 for the 7 to 14 day time interval. However, the value 0.22 is not significantly greater than other intervals due to the low sample sizes in each group. In conclusion, we were unable to definitively select a single 7 day interval of time over another as the best discriminator between S and NS. As weight loss data from longer intervals (0-14, 0-21 etc.) did not provide more predictive data than from 7 day periods, the latter are preferred because they are practically easier to obtain.

Data from the Kaplan-Meier survival curve aided the selection of periods of time to analyse the changes in bodyweight. Since only 29.3% of NS survived beyond 28 days, data for subsequent time periods (> 28 days) were less predictive. Furthermore, factors other than weight loss appeared to contribute to non-survival in horses beyond 28 days, explaining the outlying cases for NS evident in Fig 3.2. For example the horse euthanised after 4 months had reached its nadir and started to increase weight but was subsequently euthanised for acute onset intestinal ileus.

The median maximum percentage weight loss for S from first weighing was similar to that previously reported (5.2% for 34 horses; (Doxey et al., 1995a)). Clearly these data underestimate the actual weight loss occurring from disease onset. We would have hypothesised that horses lose the greatest percentage of bodyweight within the first week because of the initial reduction in mass of gastrointestinal contents. The greatest percentage total bodyweight loss for individual horses was comparable for S and NS, demonstrating that S can survive despite losing significant weight. This emphasises our recommendation that horses should not be euthanised solely on the basis of weight loss.

The median age of all horses in this study is consistent with previous reports regarding EGS (Doxey et al., 1991a, McCarthy et al., 2004). Unlike Milne et al. (1994) who found ponies significantly less likely to survive than cobs and Doxey et al. (1998) who found cobs and Thoroughbreds overrepresented in S, no breeds in the present study were associated with an increased risk of non-survival.

The duration of hospitalisation for S (34 days) was comparable for horses treated between 1991 and 1994 (median 31 days) (Doxey et al., 1995a). Horses were typically discharged from the hospital when they were consistently gaining weight whilst receiving feeds at a frequency that the owners could continue to feed at home. Obviously this is dependent on owners' willingness or desire to have the patient home before recovery is fully established. Few cases had returned to their original weight at the time of discharge. Doxey et al. (1999) did not find any correlation between duration of hospitalisation and subsequent quality of life of patients.

It is important to note that horses were never euthanised solely because of weight loss. The most prevalent single reason for euthanasia was recumbency and inability to stand due to skeletal muscle weakness. The pathogenesis of muscle weakness in CGS is unclear, but likely reflects muscle catabolism subsequent to cachexia and possibly neurogenic motor weakness (Hahn et al., 2001). It is unsurprising that weight loss was reported as the most prevalent indication when multiple indications for euthanasia were reported.

Currently, the diagnosis of grass sickness can only be definitively confirmed by histopathology of autonomic ganglia at post mortem or biopsies of the enteric nervous system collected at exploratory laparotomy (Scholes et al., 1993a, Milne et al., 2010). In the present study CGS was presumptively diagnosed after considering the nature and progression of clinical signs, history, signalment, epidemiological factors, and elimination of alternative diagnoses (Pirie et al., 2014). Diagnosing CGS by clinical examination, by clinicians familiar with the disease, has a reported accuracy of 100% (Doxey et al., 1998). Consistent with these data, all 79 NS that had a post mortem examination including histopathological examination of neural tissue were confirmed to have CGS. Consequently, we consider that diagnostic errors were unlikely to have influenced the conclusions of this study.

Survival was defined as discharge from the hospital. Whilst long-term follow up of cases was not done, previous studies indicate that the majority of CGS horses return to full athletic function (Doxey et al., 1995a, Doxey et al., 1998, Doxey et al., 1999). Doxey et al. (1995a) reported that 4 of 35 CGS cases were euthanised or died subsequent to hospital discharge.

The pathogenesis of weight loss in EGS is incompletely understood but is likely multifactorial, reflecting some of the following; lack of food intake, increased metabolic rate, cachexia, and neurogenic muscle atrophy. The authors are unaware of studies reporting the magnitude of weight loss sustained in horses following simple starvation, which could have been used to determine if weight loss is largely a consequence of reduced feed intake or if cachexia is a contributing factor. Horses experimentally deprived of food and water for 7 days had a 10% (range 8.2-10.3%) median body weight loss (Tasker, 1967). In the present study, 23% of horses lost ≥10% bodyweight over the first 7 days from the first recorded weight, suggesting that mechanisms other than simple starvation, such as cachexia, contributed to weight loss. Furthermore, since CGS cases were drinking and not completely anorexic, the weight loss would be expected to be less than reported with complete food and water deprivation. Cachexia is distinct from simple starvation, being defined as a complex metabolic syndrome associated with underlying disease and characterised by loss of muscle (Evans et al., 2008). Consistent with involvement of cachexia in grass sickness, affected horses have amino acid perturbations that resemble severe protein malnutrition, consistent with cachexia, and differing from simple starvation (McGorum and Kirk, 2001). Weight loss is a powerful independent variable that predicts mortality in human patients with cancer (Viganò et al., 2000), HIV (Wheeler et al., 1998) and the elderly in nursing homes (Sullivan et al., 2004). Excessive elaboration of proinflammatory cytokines such as interleukin (IL) 1, IL6 and tumour necrosis factor α is reported as the most common cause of cachexia in acutely ill human patients (Kotler, 2000). Proinflammatory cytokines signal an increase in the synthesis of acute phase proteins by hepatocytes. Increases in acute phase proteins such as fibrinogen, serum amyloid A (Copas et al., 2013) and haptoglobin (Milne et al., 1991) have been reported in EGS, suggesting activation of the entire inflammatory cascade and may provide further evidence that CGS horses are cachectic.

Potential errors in the data for duration of disease upon hospital admission, obtained from owners, may have resulted from delayed recognition of the subtle early signs of CGS particularly in horses at pasture. Greater sample sizes within the individual time intervals would be required for greater sensitivity and associated confidence intervals. Despite a relatively high prevalence of the disease in this area (Wylie and Proudman, 2009, Wylie et al., 2011), a significant extension in the time period for which the data was collected would be required for greater sample sizes. The percentage survival prediction curves could be used prospectively to determine their accuracy. The percentage survival prediction curves are applicable to our study population, and are likely to be less applicable to horses that do not receive intensive nursing care.

The recorded bodyweights in the study were obtained with an accurate weighbridge. To allow practitioners and owners in an ambulatory setting to use the percentage survival predictive curves, we performed a pilot study to determine which weigh tape measurement method would most accurately estimate the bodyweight (Appendix 1).

In conclusion, NS had greater bodyweight loss than S. Rapidity and magnitude of bodyweight loss were equally predictive of outcome. Percentage survival prediction curves provide objective data to aid discussion of prognosis, but greater predictive specificity with associated sensitivity is required for clinical decision making for individual horses.

3.5 Manufacturers' addresses

[a]Tru-test EziWeigh 2, Auckland, New Zealand

[b]www.r-project.org

4 Chapter Four: Conclusions

4.1 Study to increase the accuracy of equine grass sickness diagnosis using β-amyloid precursor protein immunolabelled rectal biopsies

- β-amyloid precursor protein immunoreactivity was increased in neuronal perikarya and axons in sections of CCG, ileum and rectum from EGS horses compared with controls.
- The best diagnostic predictors for discriminating EGS and control horses for the sections evaluated in this study were a weighted immunoreactivity grade exceeding 1.1, with 100% specificity and sensitivity for EGS, and the presence of at least one neuron with diffuse labelling of the entire cytoplasm (grade 3), with 95% sensitivity and 100% specificity for EGS.
- The hypothesis for the first part of the study was accepted, i.e. that immunolabelling with β-APP improved the accuracy of histological assessment of submucosal rectal biopsies for EGS diagnosis. The diagnostic accuracy is comparable to that of conventional histological examination of H&E stained ileal biopsies (Milne et al., 2010, Scholes et al., 1993a) and is significantly higher than that of conventional histological examination of two H&E stained rectal biopsies (Mair et al., 2011).
- This technique may potentially provide an accurate, minimally invasive, ante-mortem, diagnostic test for EGS. However, it should be stressed that further prospective validation of this technique is required before the use of this technique can be fully advocated for clinical decision making.

4.2 Study to increase the accuracy of prognostication for chronic grass sickness

- Consistent with the hypothesis, NS had greater bodyweight loss than S, for all time periods analysed and for the total median maximum bodyweight loss as a percentage of first weight.
- Rapidity and magnitude of bodyweight loss were equally predictive of outcome.

- The greatest percentage total bodyweight loss for individual CGS horses was comparable for S and NS. This demonstrates that S can survive despite marked weight loss, emphasising our recommendation that CGS horses should not be euthanised solely based on marked weight loss.
- Percentage survival prediction curves provide objective data, from previous outcomes, to aid discussion of prognosis for CGS horses, but greater predictive specificity and sensitivity is required for clinical decision making in individual cases.

In summary, the global aim of increasing the accuracy of diagnosis and prognostication for EGS has been achieved. Histological assessment of β-APP immunolabelled rectal biopsies aids diagnosis of EGS and bodyweight change aids prediction of survival in CGS.

5 Chapter Five: Future research

5.1 Study to increase the accuracy of equine grass sickness diagnosis using β-amyloid precursor protein immunolabelled rectal biopsies

- While the diagnostic criteria facilitated discrimination of the EGS and control rectal biopsies evaluated in this study, further prospective validation of both the grading scheme and the diagnostic criteria using a larger sample set is required.
- Further work is required to optimise differentiation of the equivocal sub-population of samples, perhaps refining the diagnostic criteria with inclusion of morphological features of neurodegeneration.
- Assessment of inter- and intra-grader correlation is warranted to determine the repeatability of the proposed grading scheme.
- A larger sample size is required to determine the effect of EGS sub-classification on neuronal β-APP immunoreactivity and its potential value for prognostication of chronic EGS cases.
- Greater numbers of pre-mortem samples should be assessed to ascertain the possible effect of pre-mortem sample collection on neuronal β-APP immunoreactivity.
- Further investigation of accelerated fixation protocols, frozen sections and rapid immunolabelling techniques is required to reduce the time requirement for sample processing thereby improving the potential use for diagnosis of acute EGS.

5.2. Study to increase the accuracy of prognostication for chronic grass sickness

- The percentage survival prediction curves should be tested prospectively to determine their accuracy. This should be done blindly after it has been determined whether the horse survives to hospital discharge or not, to avoid introduction of bias due to use of the percentage survival prediction curves for clinical decision making. For example, if a horse had lost a percentage of

bodyweight which predicts only 5% survival to discharge, this knowledge may bias the clinical decision making process towards euthanasia.

- Greater sample sizes are required for each individual time interval, to improve sensitivity and confidence intervals.
- A greater sample size of horses weighed using both weigh tapes and weigh scales is required before survival prediction curves are used with data obtained using weigh tapes.

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Appendix One

Validation of weigh tape calculations for the measurement of bodyweight in chronic equine grass sickness cases

Introduction

The recorded bodyweights in the study were obtained with an accurate weighbridge[a]. To allow practitioners and owners in an ambulatory setting to use the percentage survival predictive curves, we sought to validate and/ or calibrate the data for use with a weigh tape. Standard equine weigh tapes rely on girth measurements. However horses with chronic grass sickness (CGS) have an initial reduction in volume of gastrointestinal contents and a consequent reduction in their abdominal silhouettes, and as such it may be necessary to use different anatomical locations for weigh tape assessments of body weight in these patients.

Materials and methods

Horses admitted to the Dick Vet Equine Hospital between August 2008 and August 2010 were included in this subsidiary study. Horses were weighed on a weighbridge[a] and simultaneous bodyweights were calculated using 3 different adapted weigh tape methods; namely girth A, girth B and abdominal (Table 1). Measurements were performed at the same time each day.

Data analysis

The percentage bodyweight change over the first 7 days (from first recorded weight) was calculated and the discrepancy was plotted for each individual horse for each weigh tape method against the true weighbridge value. Bias (mean error), standard deviation and root-mean-squared error (RMSE) were calculated for each weigh tape method overall and for the individual survival statuses (survival/ non-survival).

	Bodyweight calculation (kg)	
Girth A	$\frac{G^2 \times L}{11{,}877}$	(Carroll et al., 1988)
Girth B	-421.563 + (G x 4.737)	(Marante et al., 2007)
Abdominal	-528.096 + (G x 3.101) + (U x 2.266)	(Marante et al., 2007)

Table 1. Bodyweight calculations for the 3 weigh tape methods

G = Girth (cm), measured by passing the weigh tape around the thorax immediately caudal to the elbow, and recording at the end of expiration.

L = Length (cm), measured from the point of the shoulder to the tuber ischium, following the contours of the horse's body.

U = Umbilical girth (cm), measured by passing the weigh tape around the widest aspect of the horse's abdomen, and recording at the end of expiration.

Results

The subsidiary study sample comprised 11 horses, with 4 survivors and 7 non-survivors. Median age was 5 years (interquartile range 3-10). There were 6 (54.5%) females and 5 (45.5%) geldings.

	Mean error (%)	Standard deviation	RMSE
Girth A	1.16	3.68	3.70
Girth B	-0.48	3.05	2.95
Abdominal	0.74	4.99	4.82

Table 2. Mean error, standard deviation and root-mean-squared error (RMSE) for the 3 different weigh tape measurements in comparison to the weighbridge measurement, for the percentage bodyweight change over the first 7 days from the first weight recorded.

The girth B calculation provided the best estimate of the weighbridge bodyweight with the smallest mean error, standard deviation and RMSE. This calculation underestimated the percentage bodyweight loss over the first 7 days by an average of 0.48%. The girth A calculation was a poorer estimator; overestimating the percentage bodyweight loss by an average of 1.16%. Stratifying by survival status did not provide a better estimate.

Discussion

For each weigh tape calculation some individual horse's bodyweights were underestimated and some were overestimated in comparison to the weighbridge derived weight. Consequently, a simple correction factor could not be utilised to correct for the errors in weigh tape measurements. The method most closely estimating the weighbridge weight was girth B. Whilst the mean error for the girth B calculation was small (0.48%), the standard deviation was wide, and overestimating a horse's percentage bodyweight loss over the first 7 days from the first weight by e.g. 3% could change the horse's survival prediction by 18.75%.

Horses with CGS develop a characteristic 'tucked up' abdominal silhouette and considering the gut fill of a horse is 13.5 $\pm$ 4% of bodyweight (Meyer et al., 1996) it is surprising that the abdominal weigh tape calculation did not provide the best estimate of weighbridge weight.

Only 11 horses were included in this subsidiary study and a greater sample size is required to confirm these results before the survival prediction curves are used with weigh tape bodyweights.

Manufacturers' details

[a]Tru-test EziWeigh 2, Auckland, New Zealand

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Appendix Two

Equine Veterinary Journal

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The Royal (Dick) School of Veterinary Studies

5 December 2016

Re:

JAGO, R., HANDEL, I., HAHN, C., PIRIE, R., KEEN, J., WAGGETT, B. & MCGORUM, B. 2015. Bodyweight change aids prediction of survival in chronic equine grass sickness. *Equine Veterinary Journal,* 48**,** 792-797.

Dear Dr Jago,

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Yours sincerely

Celia M Marr, BVMS, MVM, PhD, FRCVS
Editor-in-chief, Equine Veterinary Journal

Equine Veterinary Journal

Equine Veterinary Journal ISSN 0425-1644
DOI: 10.1111/evj.12551

Bodyweight change aids prediction of survival in chronic equine grass sickness

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Summary

Reasons for performing study: Objective criteria for predicting survival of chronic grass sickness cases are currently lacking.
Objectives: To determine whether the rate and/or magnitude of bodyweight change during hospitalisation of chronic grass sickness cases can provide an objective predictor of survival to discharge from hospital. Clinicians' recorded indication(s) for euthanasia were also reviewed.
Study design: Single centre retrospective observational study.
Methods: Case records of all horses admitted for management of chronic grass sickness to The Dick Vet Equine Hospital between 1998 and 2013 were analysed. Case background, survival to hospital discharge, indication(s) for euthanasia, disease duration at admission and bodyweight changes during the hospitalisation period were analysed, and data for survivors and nonsurvivors compared. Percentage weight change was calculated for 7 day intervals up to 28 days (0–7, 7–14, 14–21, 21–28 days) and for entire periods from the first weight recorded (0–7, 0–14, 0–21, 0–28 days). These results were used to estimate survival probability conditional on weight change.
Results: The study sample comprised 213 horses, with 114 survivors (53.5%) and 99 (46.5%) nonsurvivors. Compared with nonsurvivors, survivors had significantly lower median maximum bodyweight loss as a percentage of first weight (survivors 5.9%, interquartile range 1.8–13.5; nonsurvivors 12.7%, 6.4–17.3). Throughout all time periods analysed, survivors had significantly lower median bodyweight loss than nonsurvivors, but no specific time period was more predictive of survival. Highest percentages of total bodyweight loss for individual horses were comparable for survivors (36%) and nonsurvivors (37%). Survival prediction curves reporting percentage survival rates for all time periods analysed provided data to aid prediction of chronic grass sickness survival.
Conclusions: Overall, nonsurvivors had greater bodyweight loss than survivors. Rapidity and magnitude of bodyweight loss were equally predictive of outcome. Percentage survival prediction curves provide objective data to aid discussion of prognosis, but greater predictive specificity with associated sensitivity is required for clinical decision making in individual cases.

Keywords: horse; grass sickness; dysautonomia; weight loss; cachexia

Introduction

While acute and subacute grass sickness are invariably fatal, some chronic grass sickness cases survive [1–4]. Currently there are no objective criteria for predicting the outcome of chronic grass sickness cases. Accurate prediction of outcome would have welfare and economic benefits because it could reduce the number of potential survivors subjected to euthanasia and the number of nonsurvivors that are given unsuccessful intensive nursing prior to euthanasia. While a previous study demonstrated that nonsurvival was associated with severe rhinitis sicca and high subjective clinical scores based on the severity of dysphagia, anorexia, colic and reduction in intestinal sounds [1], these indices are fairly subjective. Bodyweight loss, potentially resulting from anorexia, dysphagia, rhinitis sicca, loss of taste sensation and cachexia, is a prominent feature of chronic grass sickness [5–7]. This study tested the hypothesis that magnitude and/or rate of bodyweight change from first weighing could provide an objective predictor of outcome in chronic grass sickness cases, with the magnitude and rapidity of bodyweight loss being less in survivors.

Materials and methods

Study design and data collection

A single centre retrospective observational study was conducted. Records of all horses admitted for management of chronic grass sickness to The Dick Vet Equine Hospital between 1998 and 2013, inclusive, were analysed. Information obtained from records included: age (years); sex (female/entire male/gelding); breed; survival to hospital discharge (hereafter referred to as survival status: survivor/nonsurvivor); duration of disease at admission (days) as reported by the owner; duration of hospitalisation (days); bodyweight (kg); and indication(s) for euthanasia as recorded in the final clinical report. Survival was defined as discharge from the hospital. Horses were excluded if they met the following case exclusion criteria: <2 weights were recorded or exploratory laparotomy was performed. Bodyweights were determined using a weighbridge[a] at variable times throughout the hospitalisation period.

Case management

Horses were nursed according to published guidelines [8], consisting predominantly of offering a variety of highly palatable concentrate feeds of varying consistencies. Initially, cases were fed every 2 h, with the frequency reducing as the volume of feed tolerated increased. Horses were walked daily or turned out to grass, provided this would not compromise their weakness. Analgesics and hyoscine were administered as required for episodes of colic. Some horses also received omeprazole, nonsteroidal anti-inflammatory drugs, antimicrobials, sedatives, diazepam, brotizolam, aloe vera, probiotics, dexamethasone, cisapride or acetylcysteine. Continuous flow enteral feeding and total or partial parenteral nutrition were used in a few selected cases.

Data analysis

Data were entered into an Excel spreadsheet. Categorical variables were described as percentages and chi-squared tests were used to investigate the association between categorical variables and survival status. As the continuous variables were not normally distributed they were summarised using medians and interquartile ranges (IQR). Mann–Whitney *U* tests were used to investigate their association with survival. Age was analysed as both a continuous and a categorical variable (using categories 1–2, 3, 4, 5, 6, 7, 8, 9–10 and ≥11 years).

Summary statistics were calculated for each horse, including minimum weight (as a percentage of first weight recorded), time from first weight recorded to minimum weight, duration of disease on admission and duration of hospitalisation. These were compared between survival status groups using Mann–Whitney *U* tests. Kaplan–Meier survival and time to discharge curves, from reported onset of disease, were constructed for nonsurvivors and survivors, respectively. Individual horse's bodyweights were plotted temporally, as a percentage of the first recorded weight, comparing survivors and nonsurvivors.

Referenced to day of first weight recorded, percentage bodyweight changes for each 7 day interval up to 28 days (0–7, 7–14, 14–21, 21–28 days) were used to describe the rapidity of weight loss. Percentage bodyweight changes over entire periods from the first weight (0–7, 0–14, 0–21, 0–28 days) were used to describe the overall magnitude of weight loss. If weight data were not available for the start or end of a 7-day interval, linear interpolation of the weights before and after were used to generate an estimate. Histograms were constructed from these derived data.

The percentage weight changes for all intervals for survivors and nonsurvivors were described, and compared using Mann–Whitney *U* tests. The predictive value of data from each time period for identifying nonsurvivors was described using receiver operating characteristic curves including estimation of area under the curve (AUC). An optimal cut-off was also proposed by identifying a point that would give maximum sensitivity with an estimated specificity of 1.0. A specificity of 1.0 was selected (i.e. no false positives within the study data), to minimise the possibility that a potential survivor would be subjected to euthanasia inappropriately. Estimated sensitivity was reported together with estimated 95% confidence intervals for sensitivity based on 2000 bootstrap replications of receiver operating characteristic curves.

As a potential clinical discussion tool the percentage weight changes for horses over each interval and period were summarised to estimate the probability of survival of horses grouped into ranges of percentage weight change (percentage survival prediction curves). As these estimates were based on low numbers of individual horses the uncertainty in the estimates was expressed with binomial exact confidence intervals.

As bodyweights were only recorded during the hospitalisation period and few horses were admitted on the day of onset of clinical signs, weight data were generally not available from the day of disease onset. To determine if correcting for this delay would improve predictive performance, extrapolations were performed to estimate the weight on the day of disease onset, i.e. the first day that owners recognised abnormal clinical signs. The extrapolation to day of onset used a quadratic model fitted to each horse's first 6 recorded weights, only when a weight was available ≤7 days after the onset of clinical signs, and where ≥6 weights had been recorded. These inclusion criteria for extrapolation were selected on iterative examination of fitted values under different criteria and discussion with experienced clinicians blind to the outcome of the individual horse. An extrapolated onset weight was not calculated for horses outside these criteria. Histograms and the associated quantitative data were produced for both extrapolated data (Day 0 = day of disease onset) and nonextrapolated data (Day 0 = day of first recorded weight) for all time intervals.

The R statistical system[b] was used for all statistical analyses. $P<0.05$ was used as the threshold for statistical significance. This study conformed to Standards for the Reporting of Diagnostic Accuracy guidelines where appropriate.

Results

Since 28 of the 241 horses hospitalised for management of chronic grass sickness met the case exclusion criteria, the study sample comprised 213 horses. Median age was 5 years (IQR 3.5–8). There were 96 (45.1%) females, 104 (48.8%) geldings and 13 (6.1%) entire males. Breed categories consisted of 22 (10.3%) Scottish native ponies, 31 (14.6%) other ponies, 19 (8.9%) Thoroughbreds, 42 (19.7%) cobs, 17 (8%) draught horses, 11 (5.2%) Warmbloods, 13 (6.1%) other pure breeds and 58 (27.2%) crossbreeds. No case background variables were associated with increased risk of nonsurvival.

There were 114 (53.5%) survivors and 99 (46.5%) nonsurvivors. The survival rate was (53.5%) for the 213 horses included in the study, and 49.4% for all (241) horses hospitalised for management of chronic grass sickness during the study period. All nonsurvivors were subjected to euthanasia, with none dying. For 92 of the 99 nonsurvivors, information was available describing the indications of euthanasia. The most prevalent (29.3% of nonsurvivors) single indication for euthanasia was recumbency and inability to stand. Seven cases were subjected to euthanasia for other single indications including aspiration pneumonia, functional post renal obstruction, cardiovascular collapse, diarrhoea, persistent anorexia and recurrent colic. Fifty-eight horses (63%) were subjected to euthanasia for multiple indications (Supplementary Item 1). Although weight loss (28 of 58, 48.3%) was the most prevalent indication when multiple indications for euthanasia were present, horses were never subjected to euthanasia solely because of weight loss.

There was no significant difference in age of survivors (median 5 years, IQR 3–8) and nonsurvivors (5 years, IQR 4–8), nor in duration of disease prior to hospitalisation (survivors 6 days, IQR 2–10; nonsurvivors 5 days, IQR 2–8). Survivors were hospitalised for significantly longer than nonsurvivors (survivors 34 days, IQR 22–60; nonsurvivors 14, IQR 10–25; $P<0.00001$; Fig 1). Kaplan–Meier survival curve indicated that 50% of nonsurvivors were subjected to euthanasia by 21 days and 75% by 32 days from onset of disease (Fig 1a). A time to discharge curve for survivors indicates that 50% were discharged by Day 42 from onset of disease (Fig 1b).

Horses were weighed on average every 2 days (IQR 1–3); the frequency was not significantly different between survivors and nonsurvivors. Compared with nonsurvivors, survivors had significantly lower maximum bodyweight loss as percentage of first weight (survivors 5.9%, IQR 1.8–13.5; nonsurvivors 12.7, IQR 6.4–17.3; $P<0.0001$), and a significantly earlier day of minimum weight (survivors Day 17, IQR 13–27; nonsurvivors Day 23, IQR 17–33; $P = 0.0008$). All nonsurvivors lost weight, whereas some (45.6%)

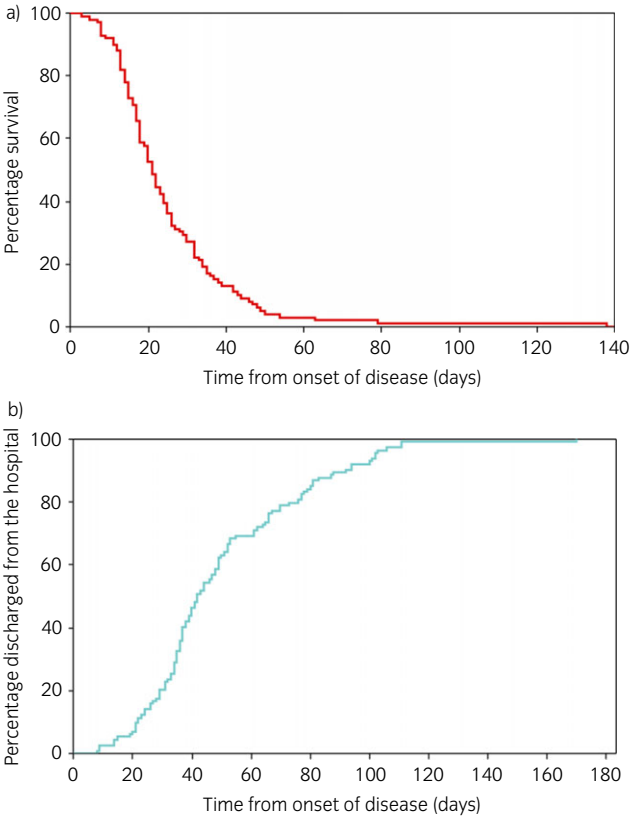

Fig 1: a) Kaplan–Meier survival curve for 99 nonsurvivors, and b) curve describing the duration of hospitalisation for 114 survivors.

survivors gained weight or shortly reached their nadir and then rapidly increased, making the overall gradient of the curve less in survivors (Fig 2). The highest percent of total bodyweight loss by individual survivors and nonsurvivors was similar (36% and 37%, respectively).

Using overall magnitude and rapidity of bodyweight change data referenced to first weight, survivors had significantly lower weight loss than nonsurvivors at all periods listed in Table 1. The greatest percentage of bodyweight loss within a 7 day period occurred between 0 and 7 days, for both survivors and nonsurvivors. The AUC was similar for all time periods, but highest between 14 and 21 days. When the cut-off was set to give a specificity of 1.0, sensitivity was fairly low for all time periods, but was greatest for the 7–14 day interval (Table 1).

The percentage bodyweight change of survivors and nonsurvivors from Day 0 (first weight) to 7 is presented in Fig 3, with histograms for other time intervals being presented in Supplementary Item 2. Percentage survival prediction curves are shown in Fig 4.

All the aforementioned results reported and later discussed are based on nonextrapolated data, with Day 0 as the first weight recorded. Only 93 horses met the criteria for extrapolation to estimate a weight at onset of disease. The predictive performance measures based on extrapolated weights were overall less informative than the measures based on first weight recorded (AUC lower for 7/8 models based on extrapolated weight compared with first weight). As extrapolation only estimates the weight at onset, it is possible that the standardising of timing it adds is overwhelmed by the error introduced by the quadratic model extrapolation.

Discussion

This is the largest study to report the outcome of chronic grass sickness cases [1–4]. The survival rates of 49.4% for all (241) horses hospitalised for management of chronic grass sickness during the study period, and of 53.5% for the 213 horses included in the study, were higher than previously reported (35.6% [1] and 42.7% [2]). The difference in percentage survival of all 241 horses compared with the 213 horses used in the study reflects the higher number of nonsurvivors in the excluded sample (23/28), probably because some were subjected to euthanasia soon after admission, prior to recording the ≥2 weights required for study inclusion.

For any given change in percentage bodyweight over any of the defined time intervals the percentage survival prediction curves report the survival rate in our study sample gathered over the last 16 years. These curves can be used to predict the survival of future cases. Obviously each clinical case should be assessed on a case by case basis – for example if the horse has concurrent aspiration pneumonia or colic, or is one of the outliers – but these values can be used as a prognostic discussion aid derived from previous outcomes, to help guide owner decisions. For example, if a horse had lost 5% bodyweight after 21 days and the owner was considering euthanasia, our data indicate that 100% of horses losing this amount of weight over this time period previously survived to discharge.

Compared with nonsurvivors, survivors had significantly lower median maximum bodyweight loss overall and for each individual time period analysed. The greatest difference in median weight loss of survivors and nonsurvivors occurred between Days 14 and 21. The greatest AUC was also for this time period; however, the confidence intervals of all areas overlap, so we cannot report that this value is significantly greater. Whilst the AUC is an objective measure of overall discrimination, both this and the difference between the medians is a population statistic and is not indicative of the best diagnostic predictor for discriminating survivors from nonsurvivors on an individual basis. With a guaranteed specificity of 1.0, the highest achievable sensitivity was 0.22 for the 7–14 day time interval. However, the value 0.22 is not significantly greater than other intervals due to the low sample sizes in each group. In conclusion, we were unable definitively to select a single 7-day interval of time over another as the best discriminator between survivors and nonsurvivors. As weight loss data from longer intervals (0–14, 0–21 etc.) did not provide more predictive data than from 7-day periods, the latter are preferred because they are easier to obtain practically.

Data from the Kaplan–Meier survival curve aided the selection of periods of time to analyse the changes in bodyweight. Since only 29.3% of nonsurvivors survived beyond 28 days, data for subsequent time periods (>28 days) were less predictive. Furthermore, factors other than weight loss appeared to contribute to nonsurvival in horses beyond 28 days, explaining the outlying cases for nonsurvivors evident in Figure 2. For example the horse subjected to euthanasia after 4 months had reached its nadir and started to increase weight but was subsequently subjected to euthanasia for acute onset intestinal ileus.

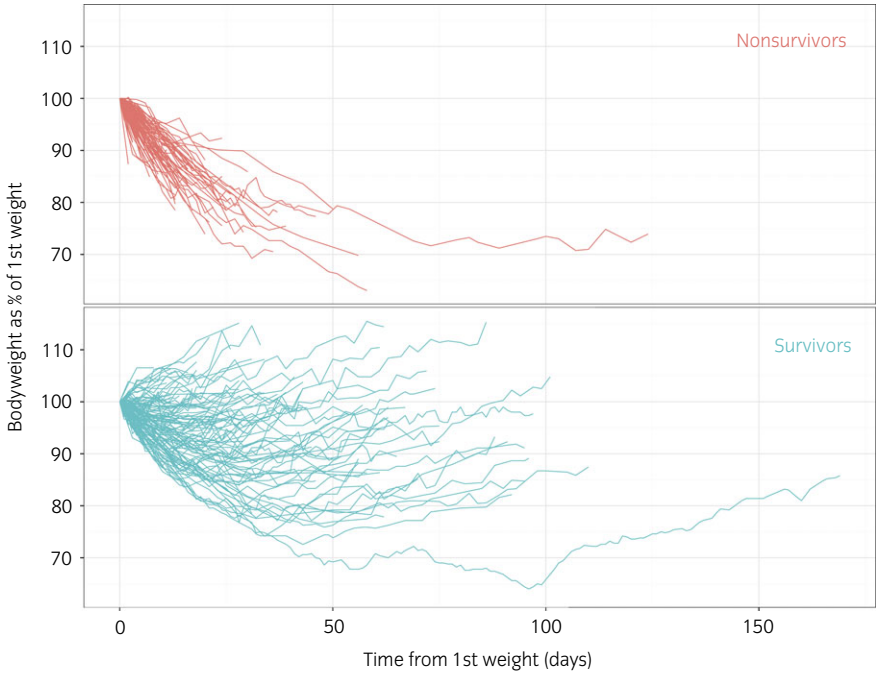

Fig 2: The temporal courses of individual horse's bodyweight as a percentage of the first recorded weight, for nonsurvivors and survivors.

TABLE 1: Median percentage bodyweight changes for various time intervals for survivors (S) and nonsurvivors (NS) from the first recorded weight, with associated areas under the receiver operating characteristic curve (AUC) and highest achievable sensitivity, when specificity was set at 1.0

Time interval (days)	S, (%) median (IQR)	NS, (%) median (IQR)	Difference between the medians of S and NS (P<0.001 for all)	AUC	95% CI for AUC	Sensitivity (specificity = 1.0)	95% CI for sensitivity
0–7	-3.4 (-6.4 to -1.1)	-7.2 (-9.6 to -5.5)	3.80	0.82	0.76–0.88	0.21	0.12–0.32
7–14	-1.8 (-4.9 to 0.2)	-5.9 (-7.4 to -4.7)	4.10	0.84	0.77–0.91	0.22	0.10–0.37
14–21	-0.02 (-2.6 to 0.9)	-5.4 (-6.6 to -5.0)	5.38	0.91	0.84–0.98	0.09	0.00–0.77
21–28	-0.2 (-2.3 to 1.7)	-5.0 (-5.5 to -2.8)	4.80	0.85	0.76–0.95	0.08	0.00–0.25
0–7	-3.4 (-6.4 to -1.1)	-7.2 (-9.6 to -5.5)	3.80	0.82	0.76–0.88	0.21	0.12–0.32
0–14	-6.1 (-10.3 to -0.9)	-12.3 (-15.1 to -10.1)	6.20	0.83	0.76–0.90	0.20	0.10–0.37
0–21	-7.5 (-12.9 to -2.8)	-16.6 (-18.7 to -15.1)	9.10	0.88	0.81–0.95	0.18	0.05–0.36
0–28	-9.2 (-15.5 to -2.2)	-19.9 (-22.8 to -18.1)	10.70	0.87	0.78–0.96	0.25	0.08–0.50

IQR = interquartile range; CI = confidence interval.

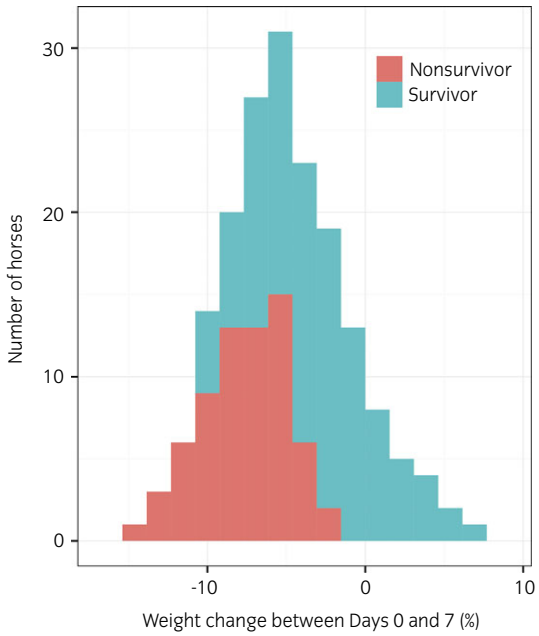

Fig 3: Histogram demonstrating the percentage bodyweight change of survivors and nonsurvivors from Day 0 (first weight) to Day 7. For example 13 horses lost 2% bodyweight and all these were survivors. Six horses lost 12% bodyweight and all were nonsurvivors. Thirty-one horses lost 6% bodyweight and these horses were a mixture of survivors and nonsurvivors.

The median maximum percentage weight loss for survivors from first weighing was similar to that previously reported (5.2% for 34 horses; [2]). Clearly these data underestimate the actual weight loss occurring from disease onset. We would have hypothesised that horses lose the greatest percentage of bodyweight within the first week because of the initial reduction in mass of gastrointestinal contents. The greatest percentage total bodyweight loss for individual horses was comparable for survivors and nonsurvivors, demonstrating that survivors can survive despite losing significant weight. This emphasises our recommendation that horses should not be subjected to euthanasia solely on the basis of weight loss.

The median age of all horses in this study is consistent with previous reports regarding equine grass sickness [9,10]. Unlike Milne *et al.* [1] who found ponies significantly less likely to survive than cobs and Doxey *et al.* [3] who found cobs and Thoroughbreds overrepresented in survivors, no breeds in the present study were associated with an increased risk of nonsurvival.

The duration of hospitalisation for survivors (34 days) was comparable for horses treated between 1991 and 1994 (median 31 days) [2]. Horses were typically discharged from the hospital when they were consistently gaining weight while receiving feeds at a frequency that the owners could continue to feed at home. Obviously this is dependent on owners' willingness or desire to have the case home before recovery is fully established. Few cases had returned back to their original weight at the time of discharge. Doxey *et al.* [4] did not find any correlation between duration of hospitalisation and subsequent quality of life of horses.

It is important to note that horses were never subjected to euthanasia solely because of weight loss. The most prevalent single reason for euthanasia was recumbency and inability to stand due to skeletal muscle weakness. The pathogenesis of muscle weakness in chronic grass sickness is unclear, but is likely to reflect muscle catabolism subsequent to cachexia and possibly neurogenic motor weakness [11]. It is unsurprising that weight loss was reported as the most prevalent indication when multiple indications for euthanasia were reported.

Currently, the diagnosis of grass sickness can only be definitively confirmed by histopathology of autonomic ganglia at *post mortem* or biopsies of the enteric nervous system collected at exploratory laparotomy [12,13]. In the present study chronic grass sickness was presumptively diagnosed after considering the nature and progression of clinical signs, history, case background, epidemiological factors and elimination of alternative diagnoses [7]. Diagnosing chronic grass sickness by clinical examination, by clinicians familiar with the disease, has a reported accuracy of 100% [3]. Consistent with these data, all 79 nonsurvivors that had a *post mortem* examination including histopathological examination of neural tissue were confirmed to have chronic grass sickness. Consequently we consider that diagnostic errors were unlikely to have influenced the conclusions of this study.

Survival was defined as discharge from the hospital. While long-term follow-up of cases was not done, previous studies indicate that the majority of chronic grass sickness horses return to full athletic function [2–4]. Doxey *et al.* [2] reported that 4 of 35 chronic grass sickness cases were subjected to euthanasia or died subsequent to hospital discharge.

The pathogenesis of weight loss in equine grass sickness is incompletely understood but is likely to be multifactorial, reflecting some of the following; lack of food intake; increased metabolic rate; cachexia and neurogenic muscle atrophy. The authors are unaware of studies reporting the magnitude of weight loss sustained in horses following simple starvation, which could have been used to determine if weight loss is largely a consequence of reduced feed intake or if cachexia is a contributing factor. Horses experimentally deprived of food and water for 7 days had a 10% (range 8.2–10.3%) median body weight loss [14]. In the present study, 23% of horses lost ≥10% bodyweight over the first 7 days from the first recorded weight, suggesting that mechanisms other than simple starvation, such as cachexia, contributed to weight loss. Furthermore, since chronic grass sickness cases were drinking and not completely anorexic, the weight loss would be expected to be less than reported with complete food

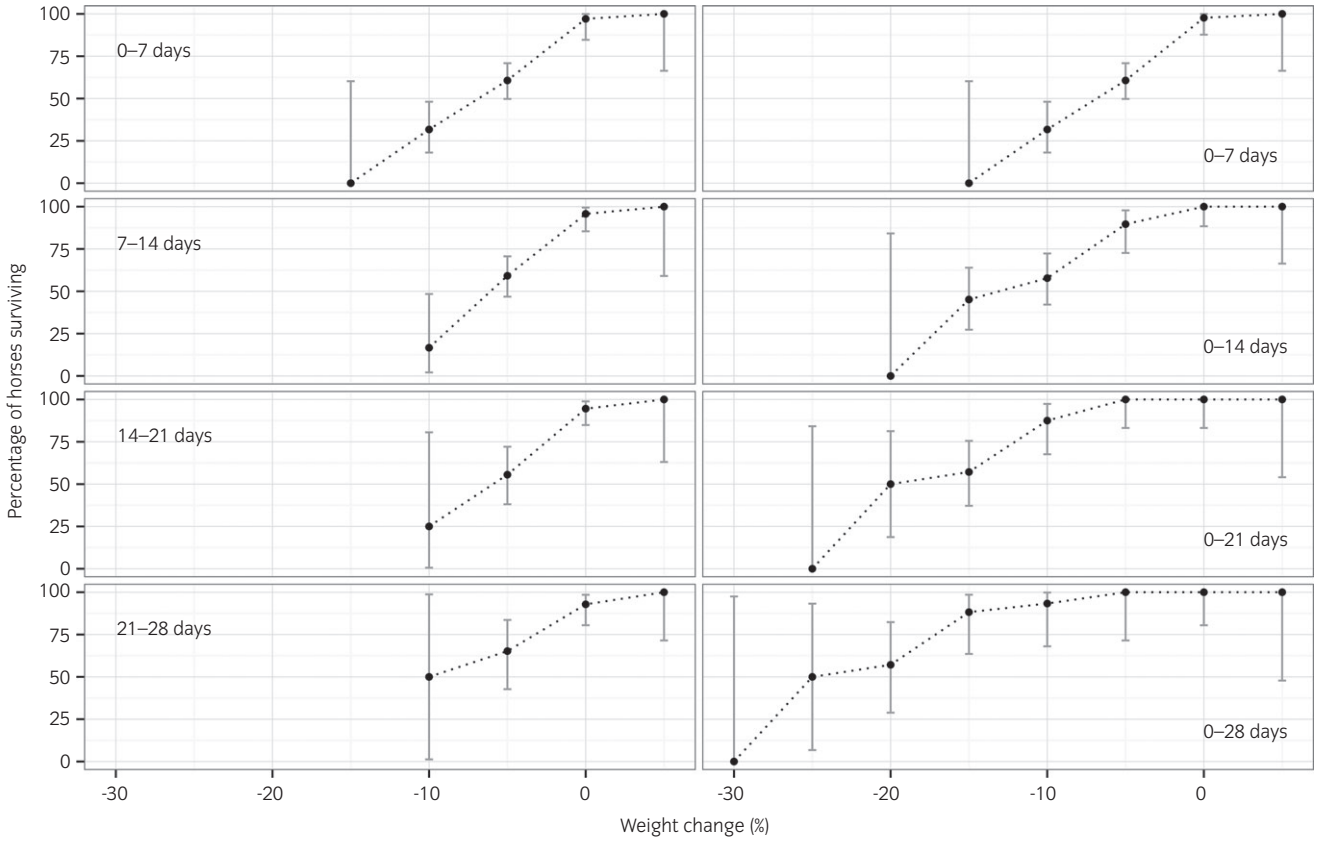

Fig 4: Percentage survival prediction curves for each 7 day period and each period from the time of first weight up to 28 days, in 7 day increments.

and water deprivation. Cachexia is distinct from simple starvation, being defined as a complex metabolic syndrome associated with underlying disease and characterised by loss of muscle [15]. Consistent with involvement of cachexia in grass sickness, affected horses have amino acid perturbations that resemble severe protein malnutrition, consistent with cachexia, and differing from simple starvation [16]. Weight loss is a powerful independent variable that predicts mortality in human patients with cancer [17] or human immunodeficiency virus [18], and the elderly in nursing homes [19]. Excessive elaboration of proinflammatory cytokines such as interleukins 1 and 6 and tumour necrosis factor α is reported as the most common cause of cachexia in acutely ill human patients [20]. Proinflammatory cytokines signal an increase in the synthesis of acute phase proteins by hepatocytes. Increases in acute phase proteins such as fibrinogen, serum amyloid A [21] and haptoglobin [22] have been reported in equine grass sickness, suggesting activation of the entire inflammatory cascade and may provide further evidence that chronic grass sickness horses are cachectic.

Potential errors in the data for duration of disease upon hospital admission, obtained from owners, may have resulted from delayed recognition of the subtle early signs of chronic grass sickness particularly in horses at pasture. Greater sample sizes within the individual time intervals would be required for greater sensitivity and associated confidence intervals. Despite a relatively high prevalence of the disease in this area [6,23], a significant extension in the time period for which the data were collected would be required for greater sample sizes. The percentage survival prediction curves could be used prospectively to determine their accuracy. The percentage survival prediction curves are applicable to our study population, and are likely to be less applicable to horses, that do not receive intensive nursing care.

The recorded bodyweights in the study were obtained with an accurate weighbridge. To allow practitioners and owners in an ambulatory setting to use the percentage survival predictive curves, we performed a pilot study to determine which weigh tape measurement method would most accurately estimate the bodyweight (Supplementary Item 3).

In conclusion, nonsurvivors had greater bodyweight loss than survivors. Rapidity and magnitude of bodyweight loss were equally predictive of outcome. Percentage survival prediction curves provide objective data to aid discussion of prognosis, but greater predictive specificity with associated sensitivity is required for clinical decision making for individual horses.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Research ethics committee oversight not required by this journal: retrospective study of clinical records. Explicit owner informed consent for inclusion of animals in this study was not stated.

Source of funding

The Equine Grass Sickness Fund provided funding for the nursing care of all cases.

Acknowledgements

The authors thank all clinicians and support staff at the Dick Vet Equine Hospital for their contribution to patient care, and the veterinary surgeons

who referred the cases. We especially acknowledge the grooms and nurses who spent considerable time and effort nursing patients. We thank Dr Darren Shaw for statistical advice.

Authorship

B.C. McGorum, I. Handel and R.C. Jago were responsible for study design, interpretation and manuscript preparation. I. Handel was responsible for statistical analysis. R.C. Jago collected data and contributed to statistical analysis. C.N. Hahn contributed to study design. B.C. McGorum, R.S. Pirie and J.A. Keen provided the majority of cases. B.E. Waggett contributed to data collection. All authors reviewed the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Item 1: Frequency of reported indications for euthanasia in 58 horses that had >1 indication for euthanasia.

Supplementary Item 2: Histograms demonstrating the percentage bodyweight change of survivors and nonsurvivors for each 7 day period and each period from the time of first weight up to 28 days, in 7 day increments.

Supplementary Item 3: Validation of weigh tape calculations for the measurement of body weight in chronic equine grass sickness cases.